

Appendix A: U.S. Patent No. 5,716,981



US005716981A

United States Patent [19]

Hunter et al.

[11] Patent Number: 5,716,981
[45] Date of Patent: Feb. 10, 1998

- [54] ANTI-ANGIOGENIC COMPOSITIONS AND METHODS OF USE
- [75] Inventors: William L. Hunter; Lindsay S. Machan, both of Vancouver; A. Larry Arsenault, Paris, all of Canada
- [73] Assignee: Angiogenesis Technologies, Inc., Vancouver, Canada
- [21] Appl. No.: 478,203
- [22] Filed: Jun. 7, 1995

Related U.S. Application Data

- [60] Division of Ser. No. 417,160, Apr. 3, 1995, abandoned, which is a continuation-in-part of Ser. No. 94,536, Jul. 19, 1993, abandoned.

[30] Foreign Application Priority Data

- Jul. 19, 1994 [WO] WIPO PCT/CA94/00373
- [51] Int. Cl.⁶ A61K 31/335; A61M 29/00
- [52] U.S. Cl. 514/449; 128/898; 526/304; 528/421; 604/53; 604/20; 604/21; 604/96; 604/269; 606/198; 623/12
- [58] Field of Search 604/53, 20, 21, 604/96, 269; 128/898; 514/449; 606/198; 623/12; 528/421; 526/304

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[57] ABSTRACT

The present invention provides compositions comprising an anti-angiogenic factor, and a polymeric carrier. Representative examples of anti-angiogenic factors include Anti-Invasive Factor, Retinoic acids and derivatives thereof, and paclitaxel. Also provided are methods for embolizing blood vessels, and eliminating biliary, urethral, esophageal, and tracheal/bronchial obstructions.

18 Claims, 75 Drawing Sheets

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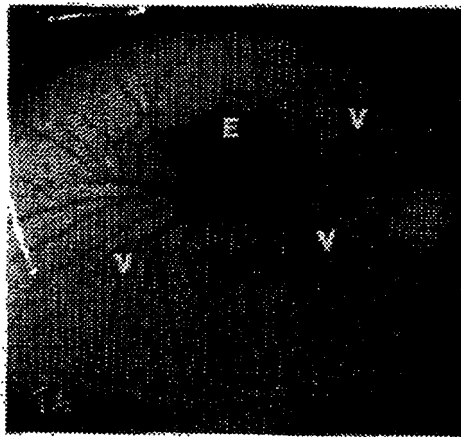


Fig. 1A

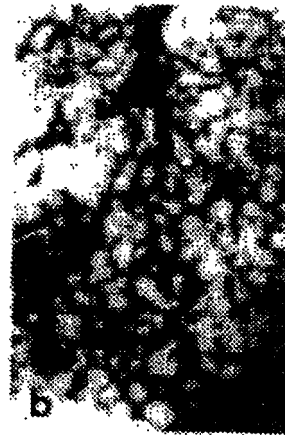


Fig. 1B

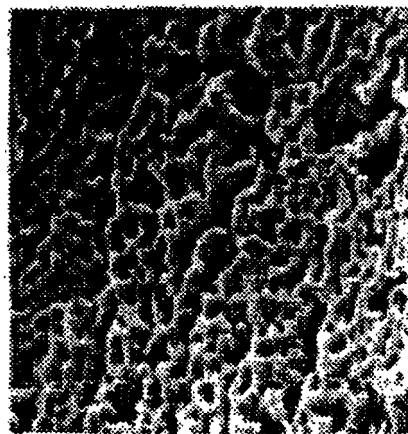
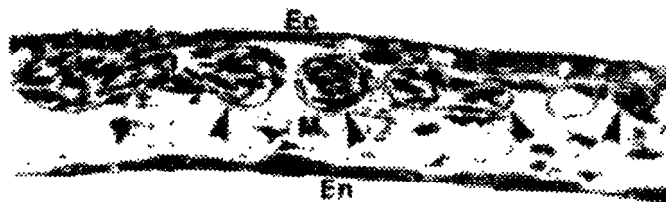


Fig. 1C



d

Fig. 1D



Fig. 1E



Fig. 2A



Fig. 2B



Fig. 2C

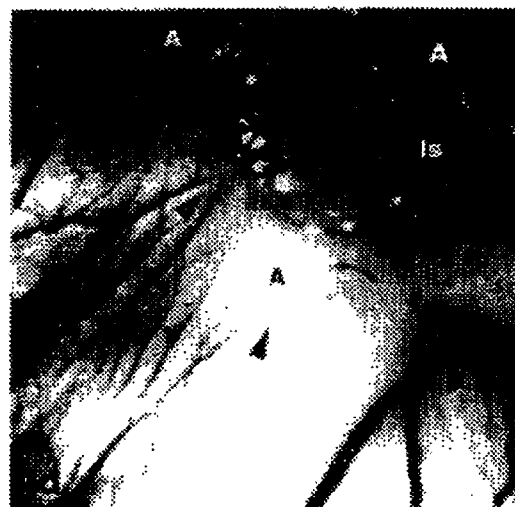


Fig. 2D

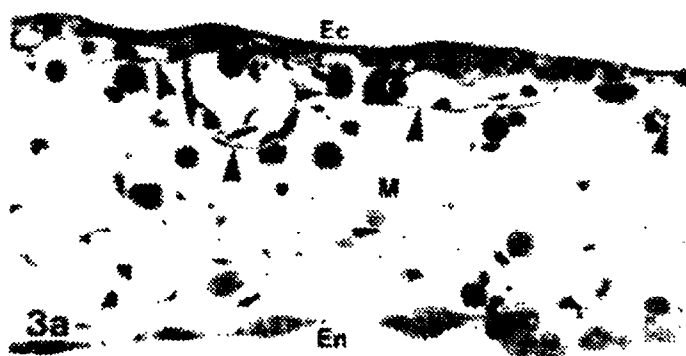


Fig. 3A

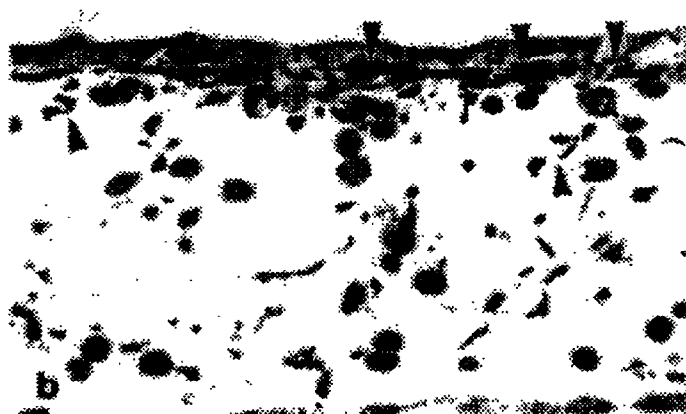


Fig. 3B



Fig. 3C

Fig. 4A



Fig. 4B

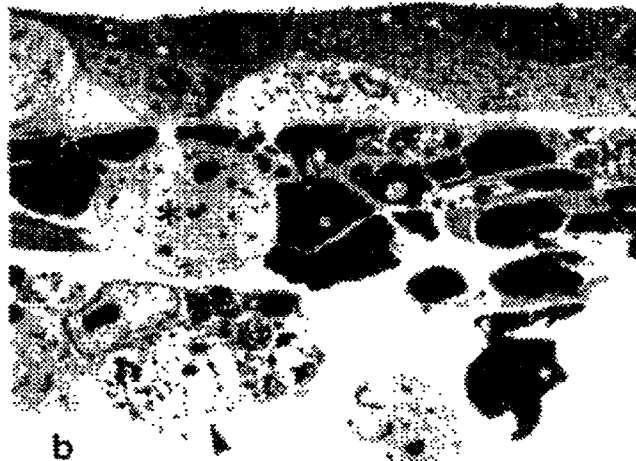
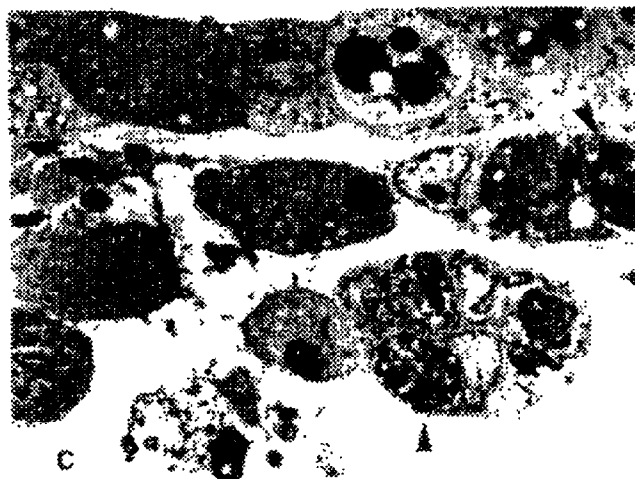
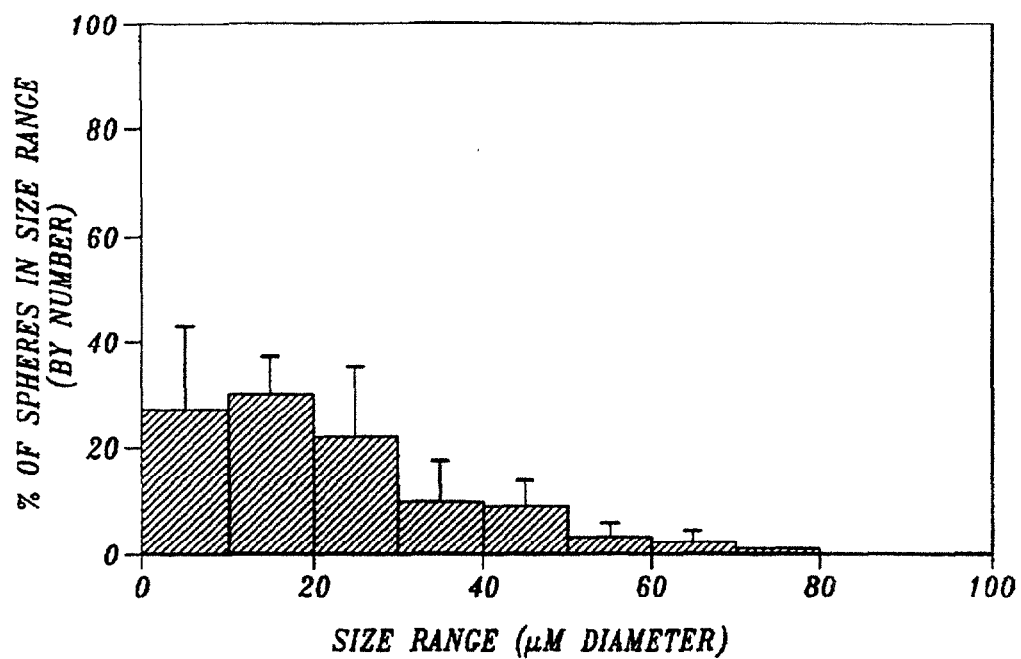
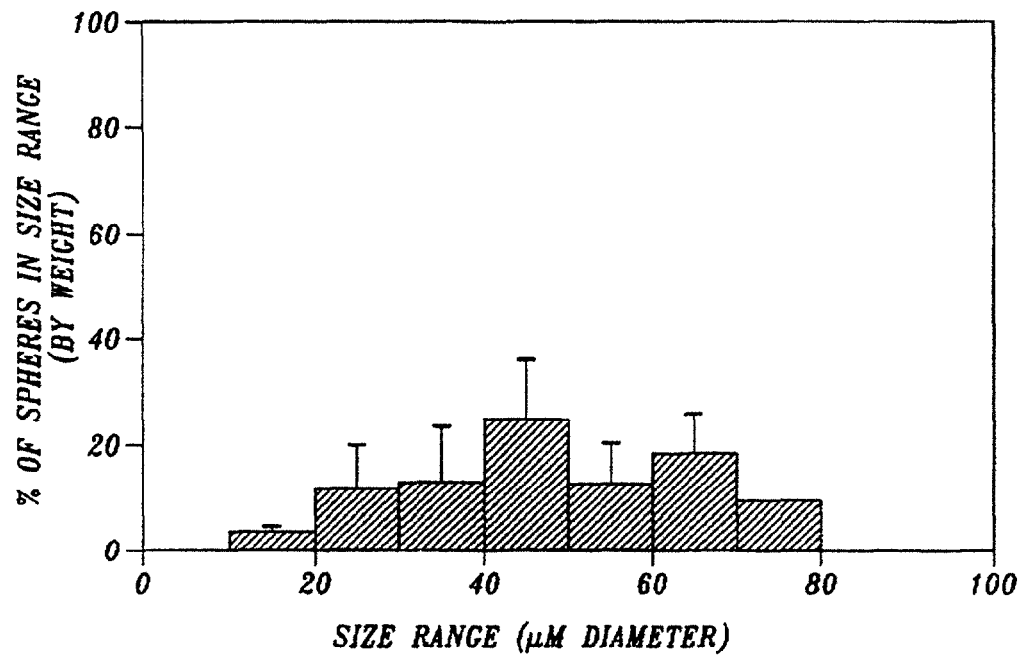
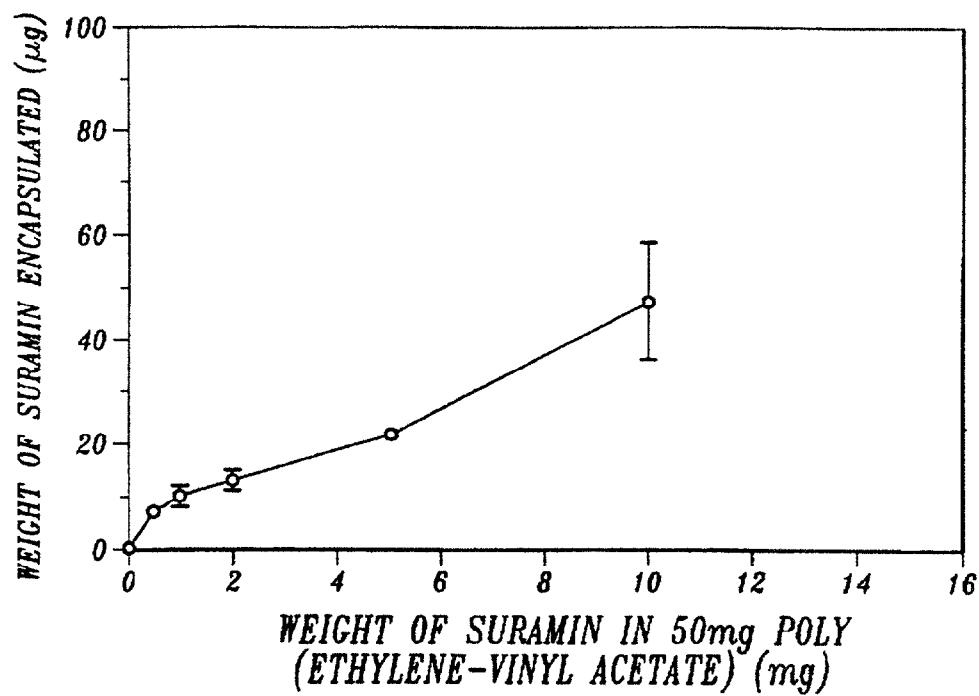
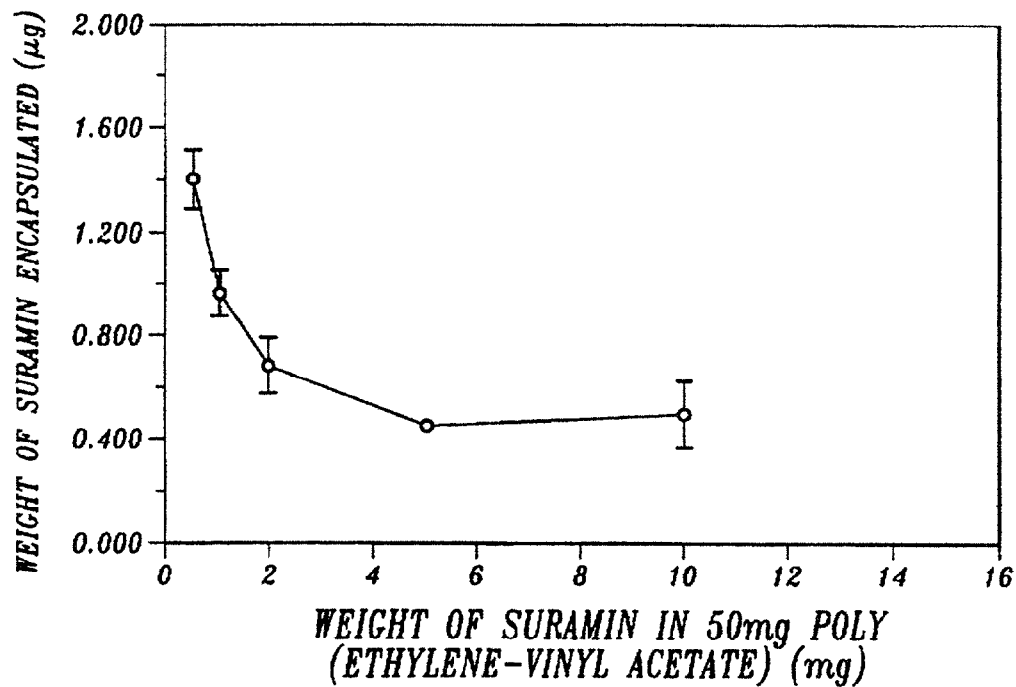
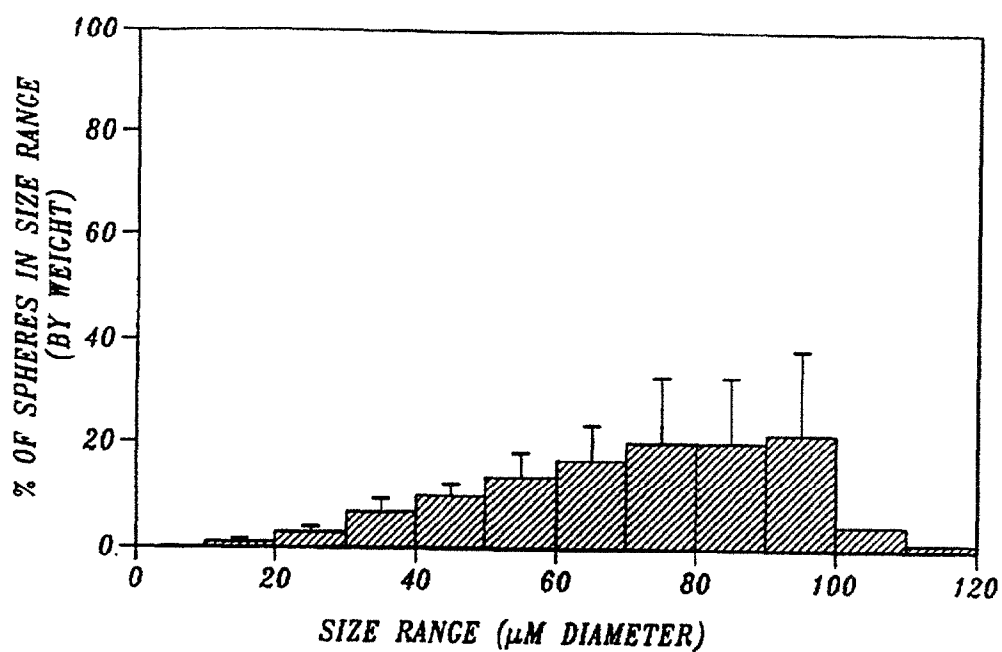
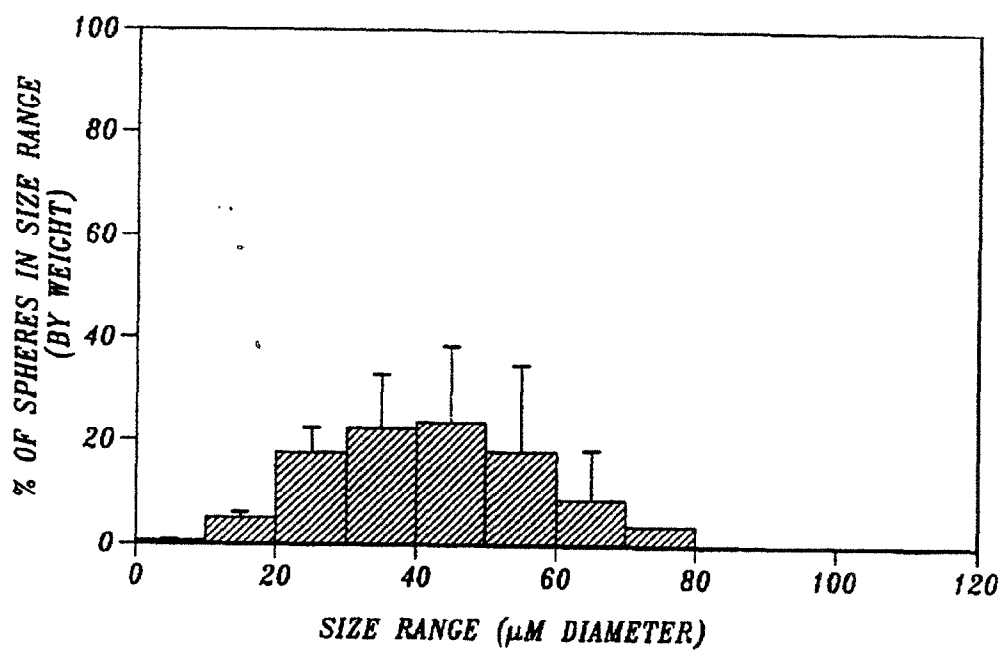


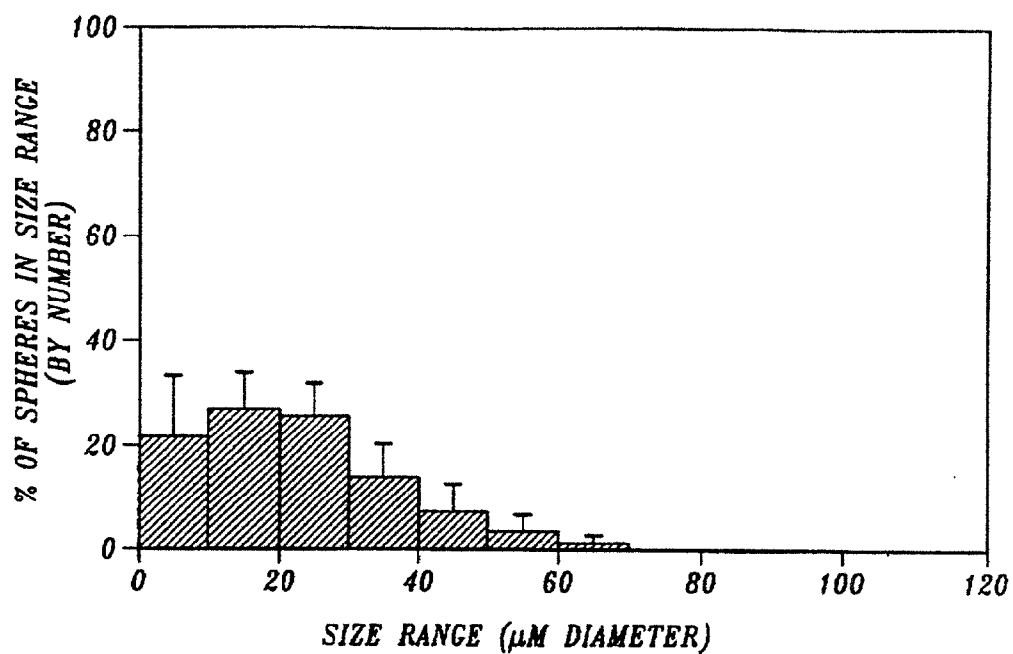
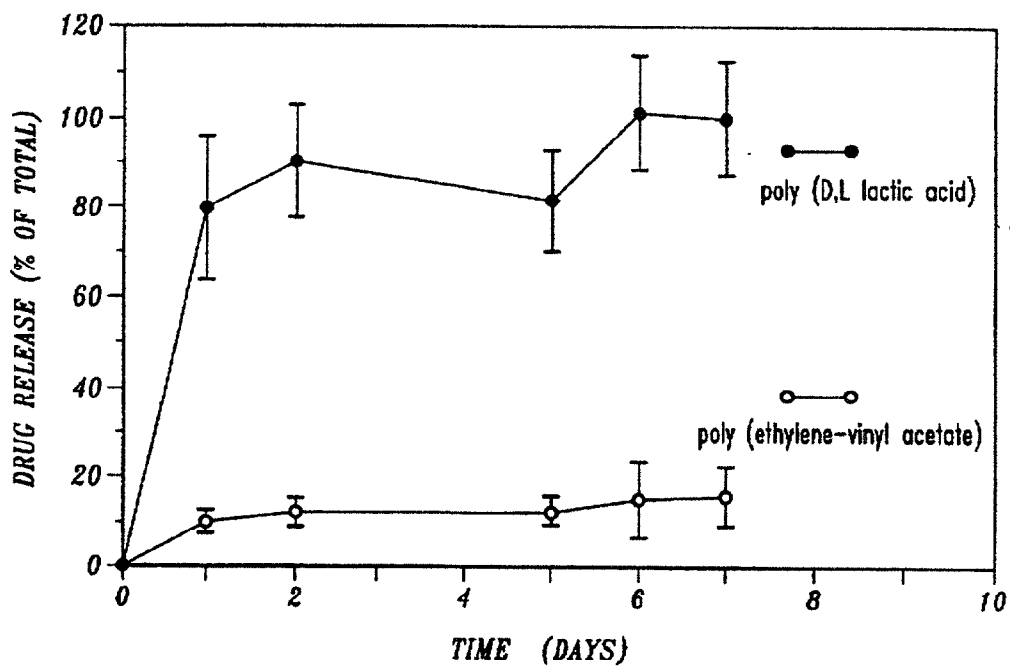
Fig. 4C

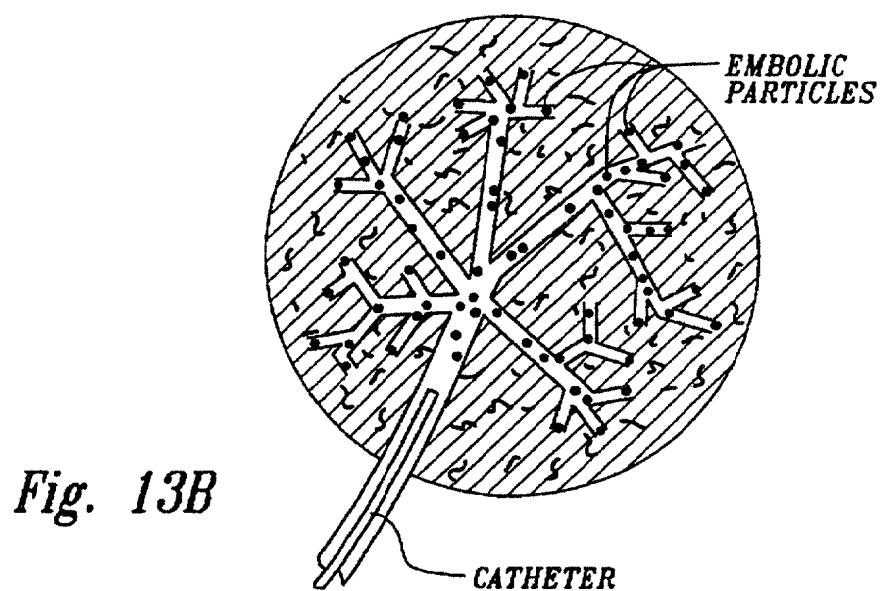
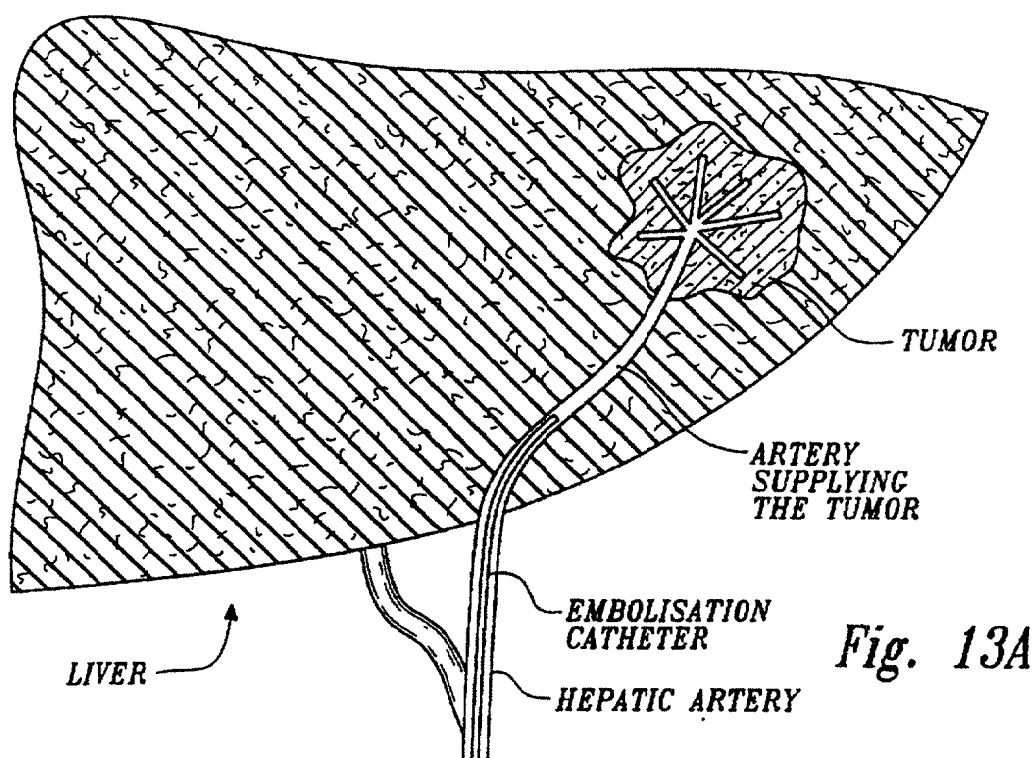


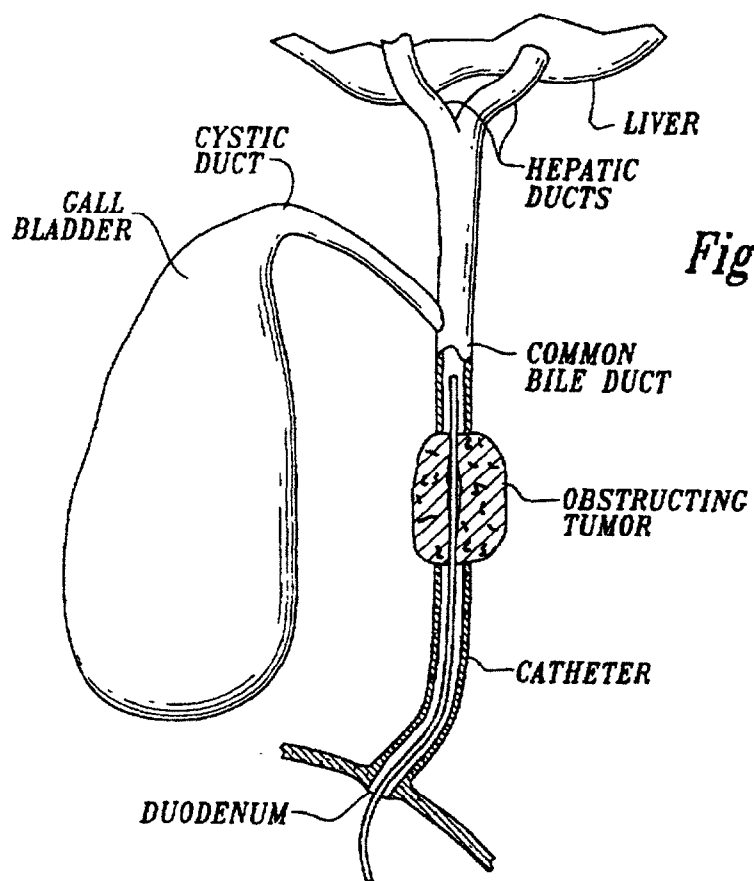
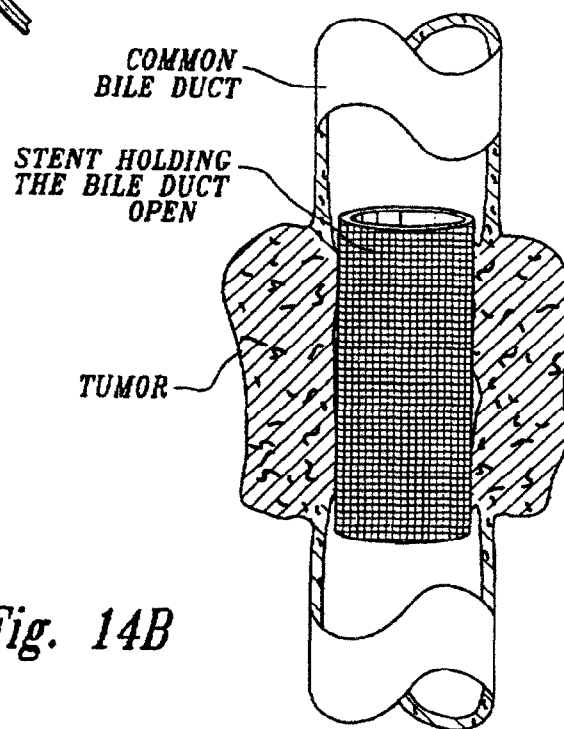
*Fig. 5**Fig. 6*

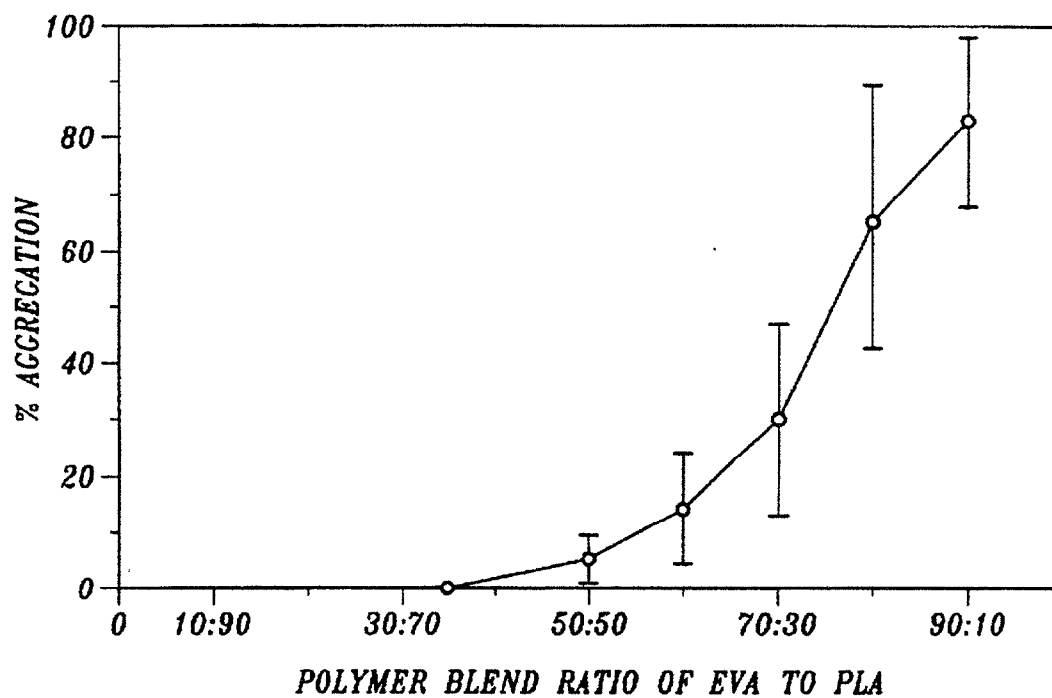
*Fig. 7**Fig. 8*

*Fig. 9**Fig. 10*

*Fig. 11**Fig. 12*



*Fig. 14A**Fig. 14B*

*Fig. 15A*

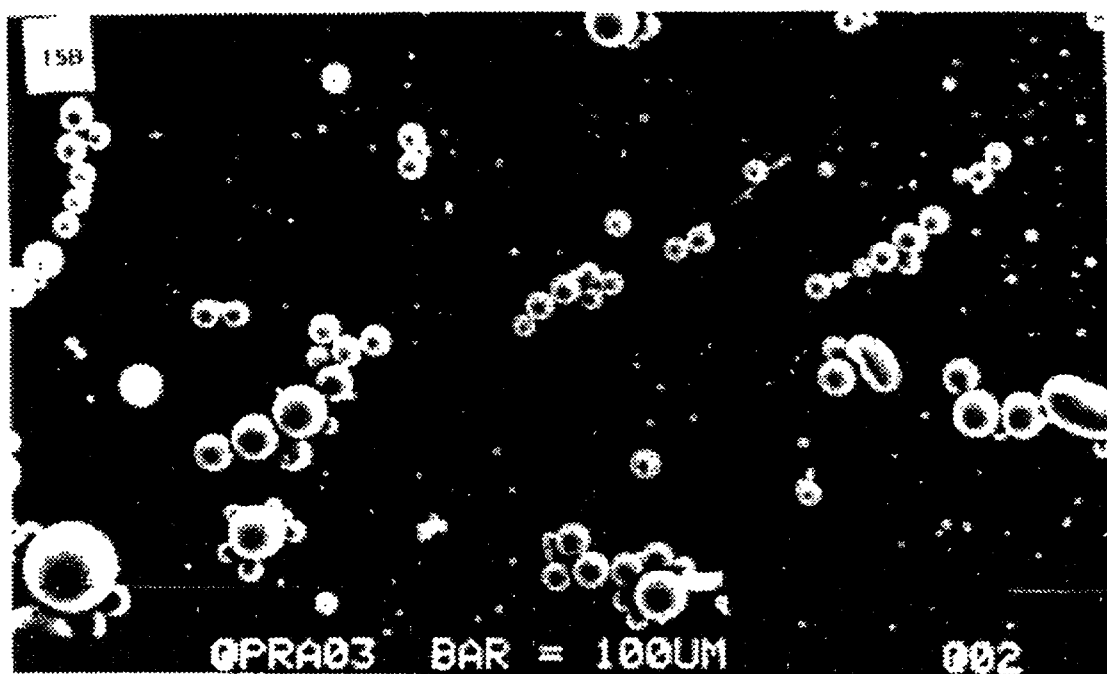
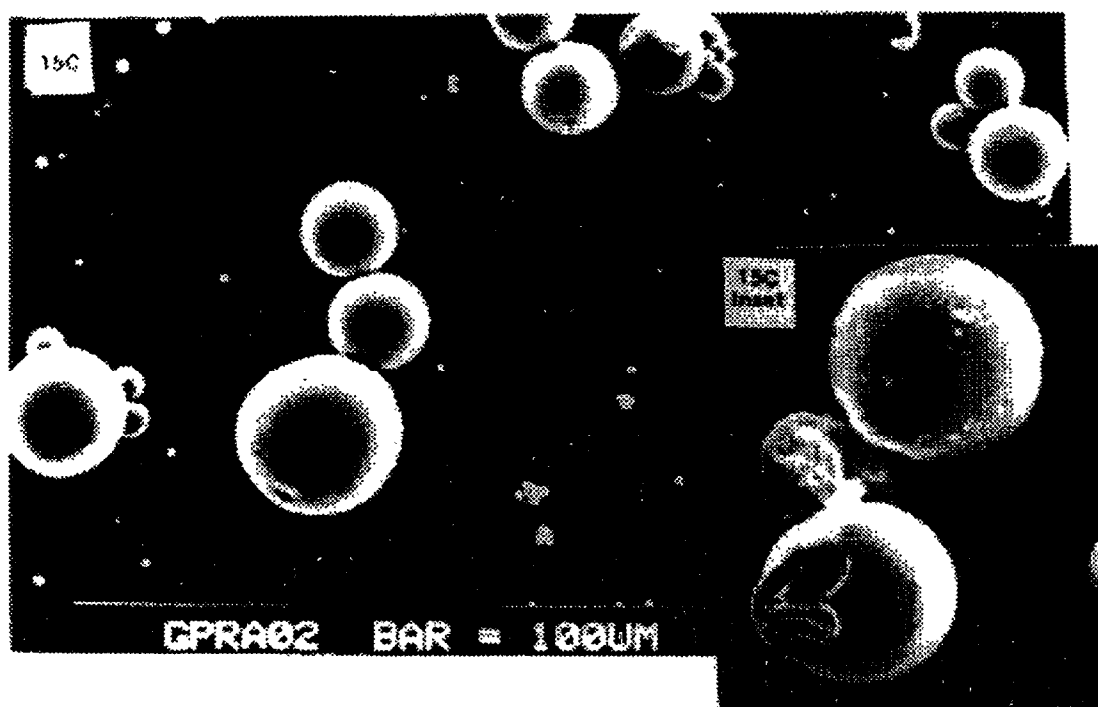


Fig. 15B

*Fig. 15C*

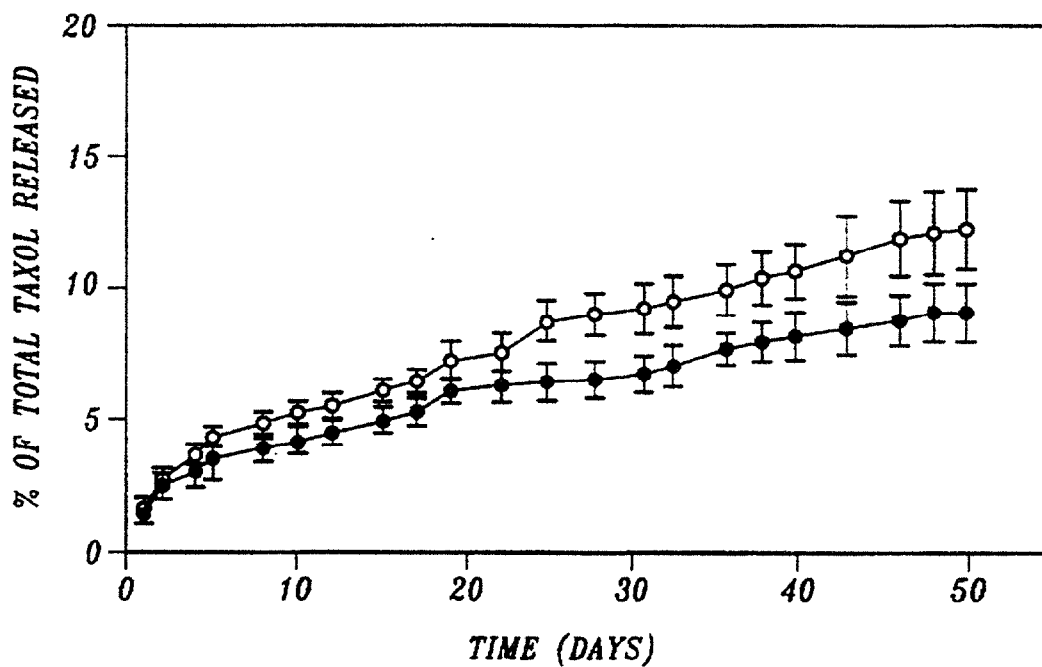
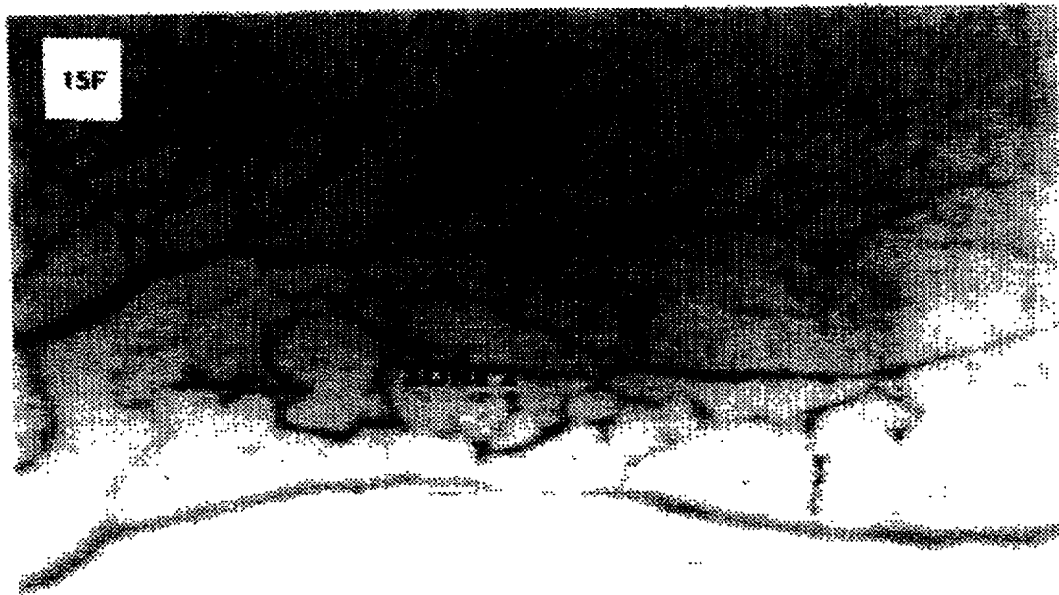
*Fig. 15D*



Fig. 15E



ZONE 1

MS

Fig. 15F

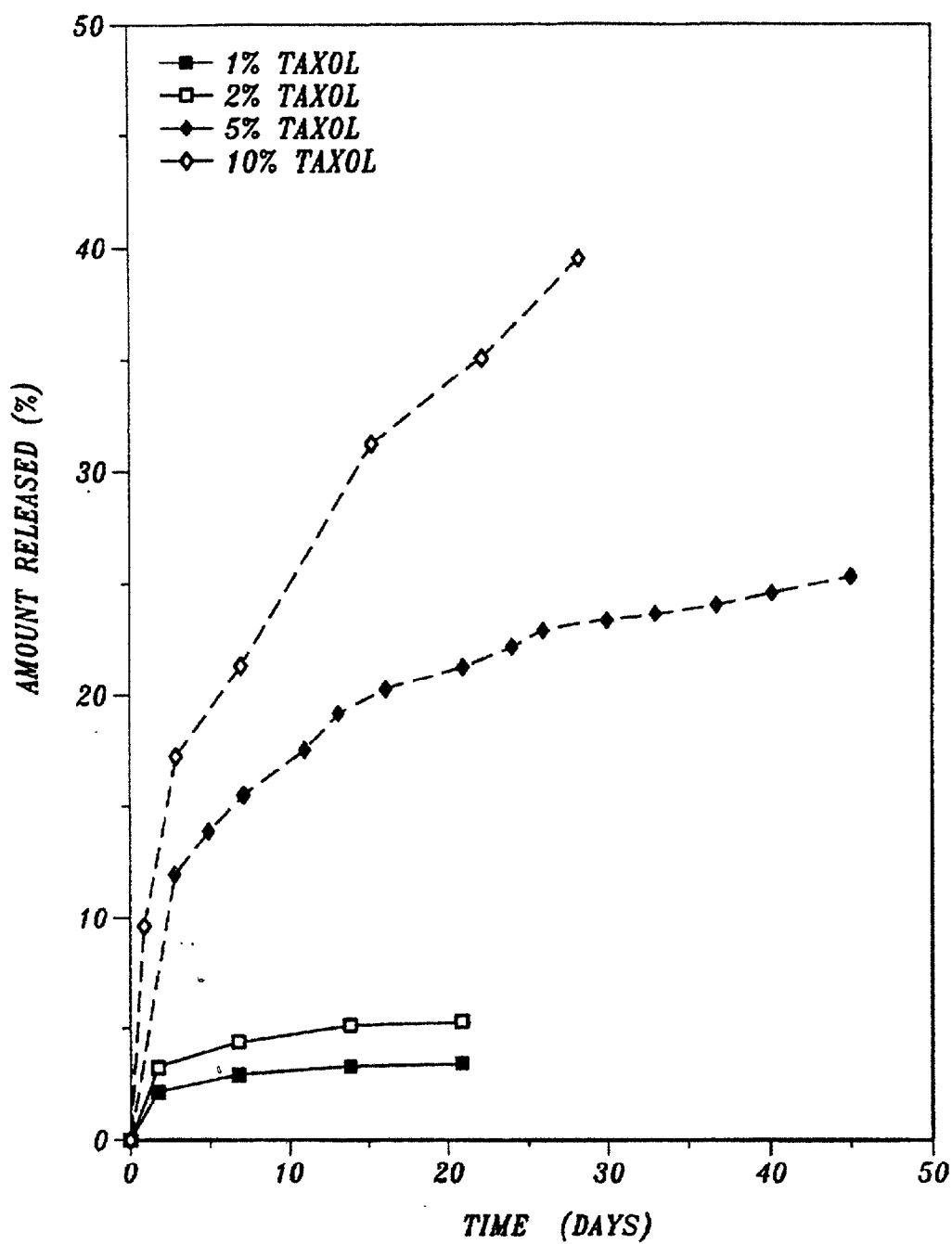
*Fig. 16A*



Fig. 16B

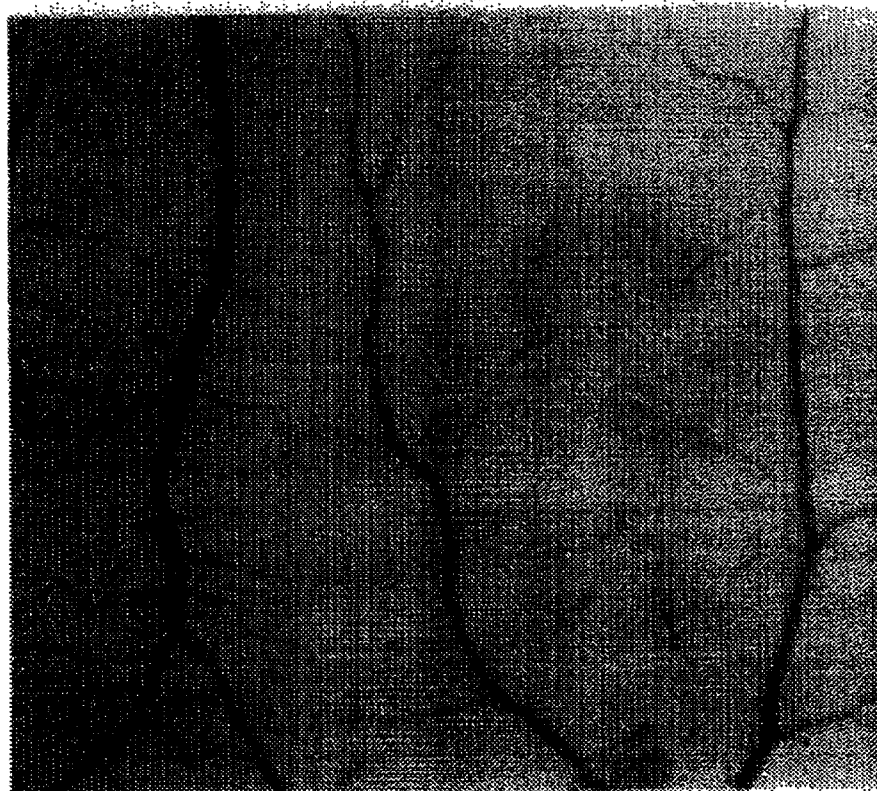
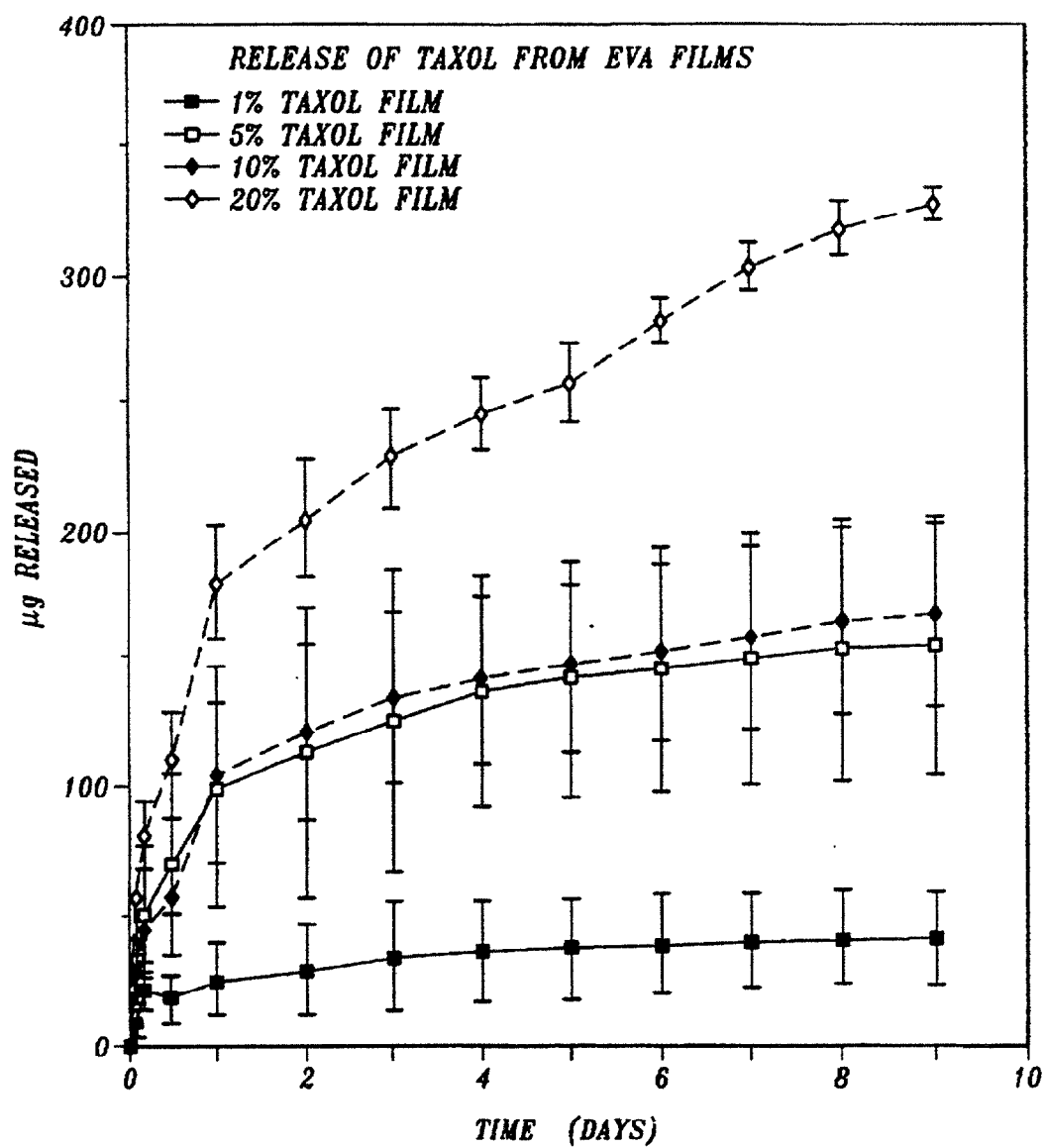
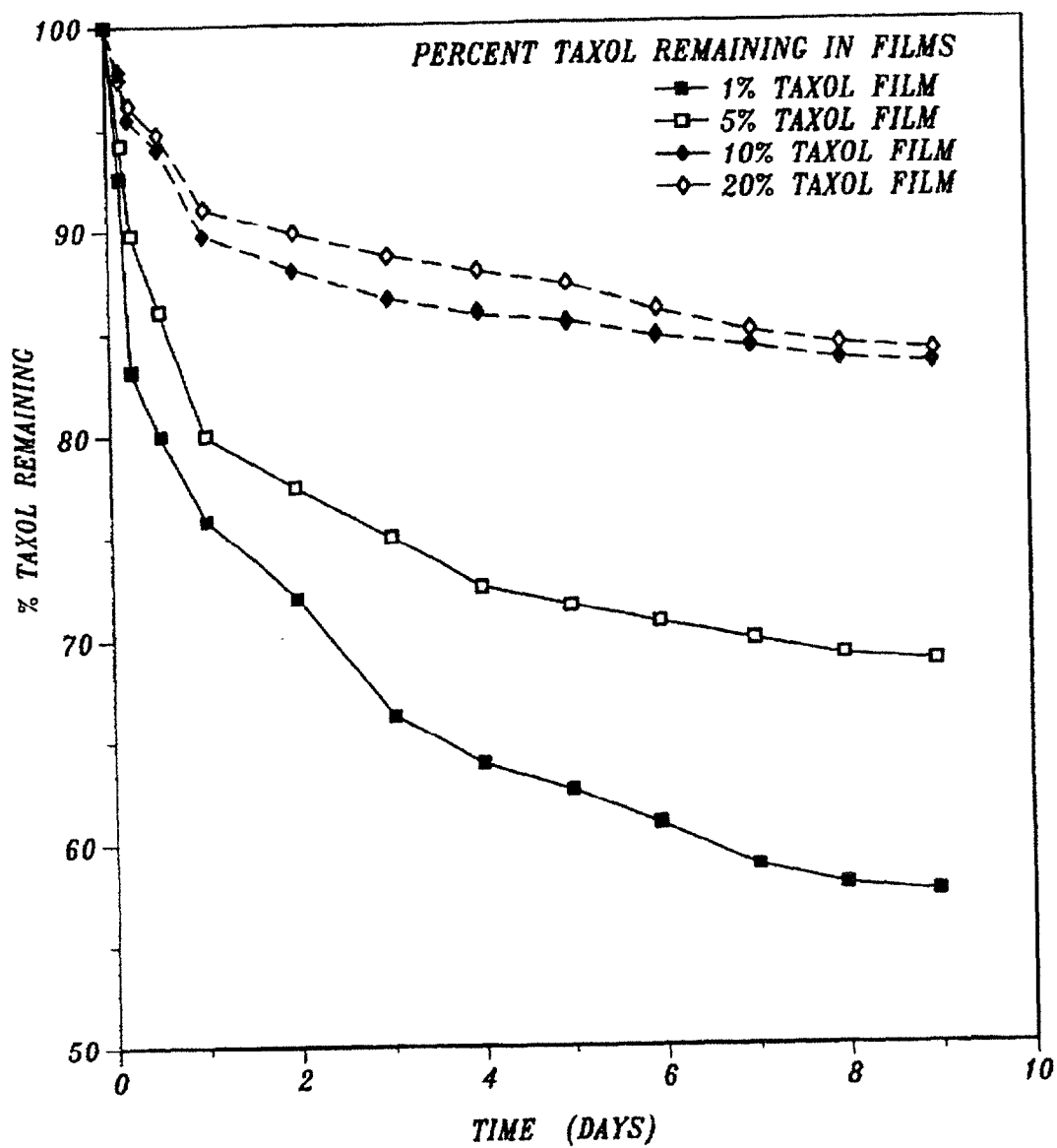
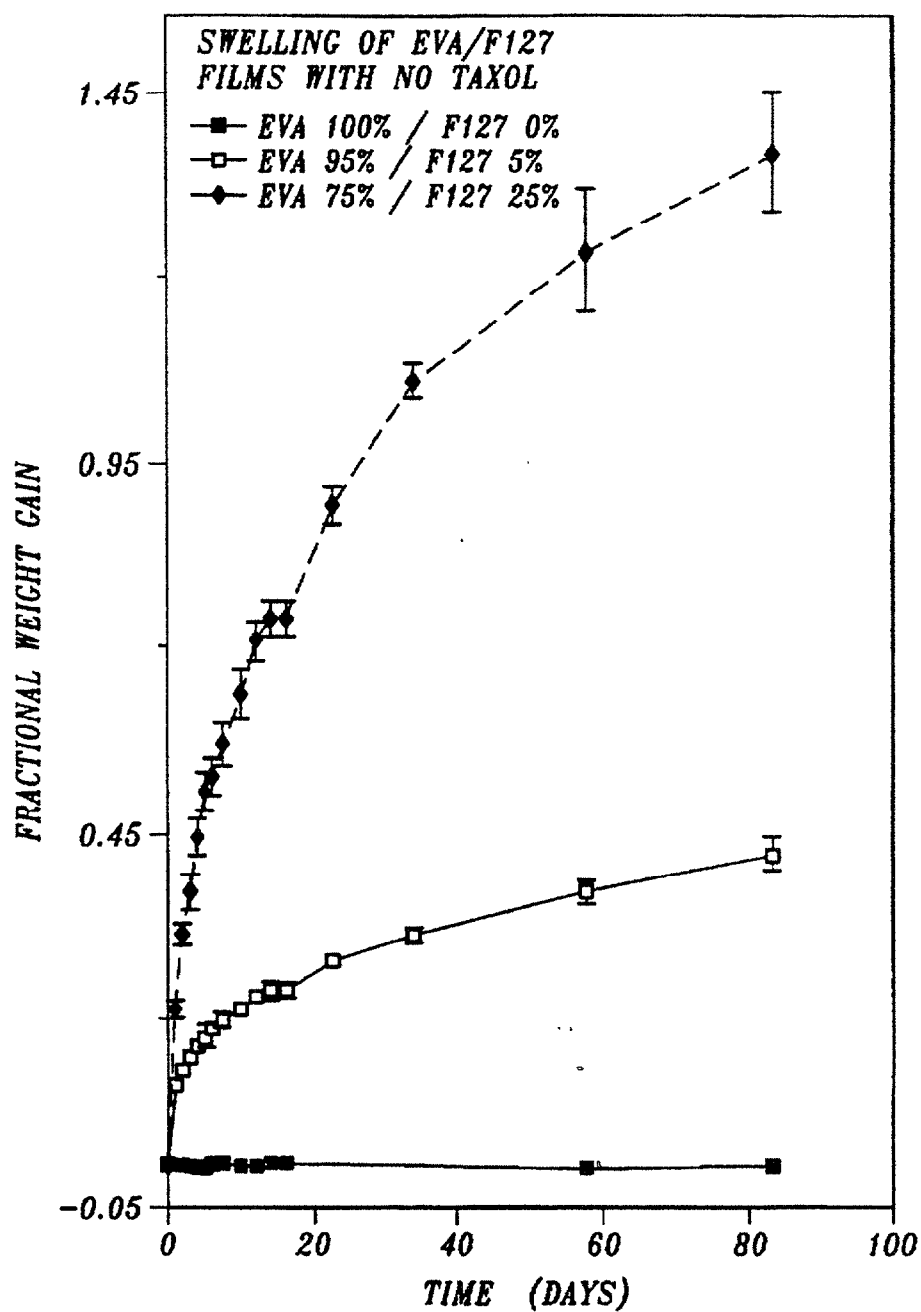
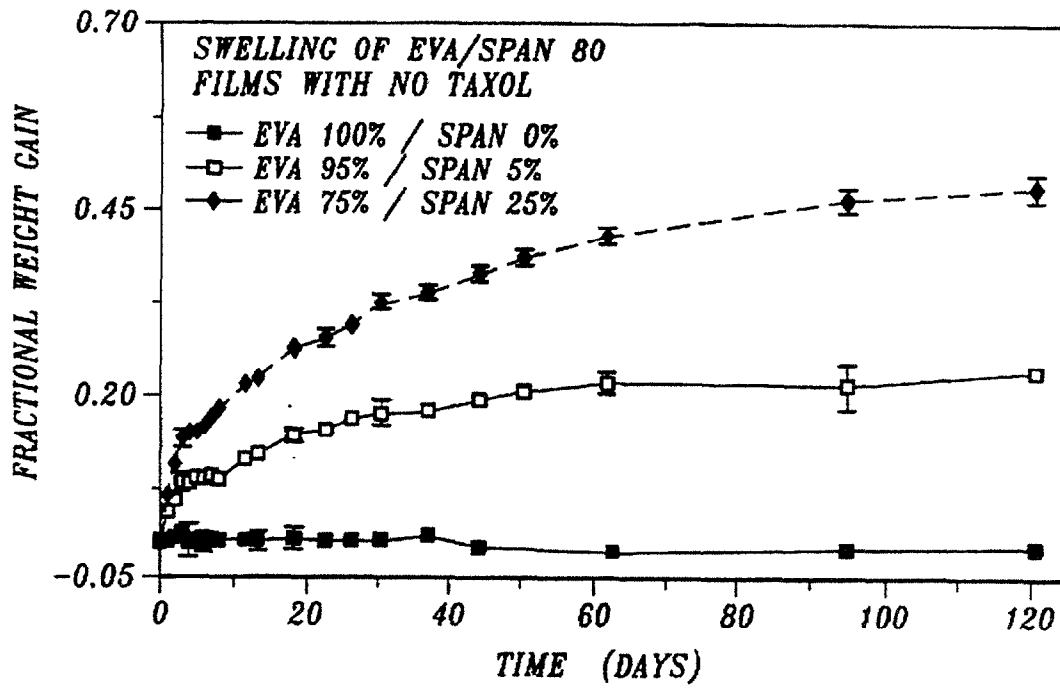


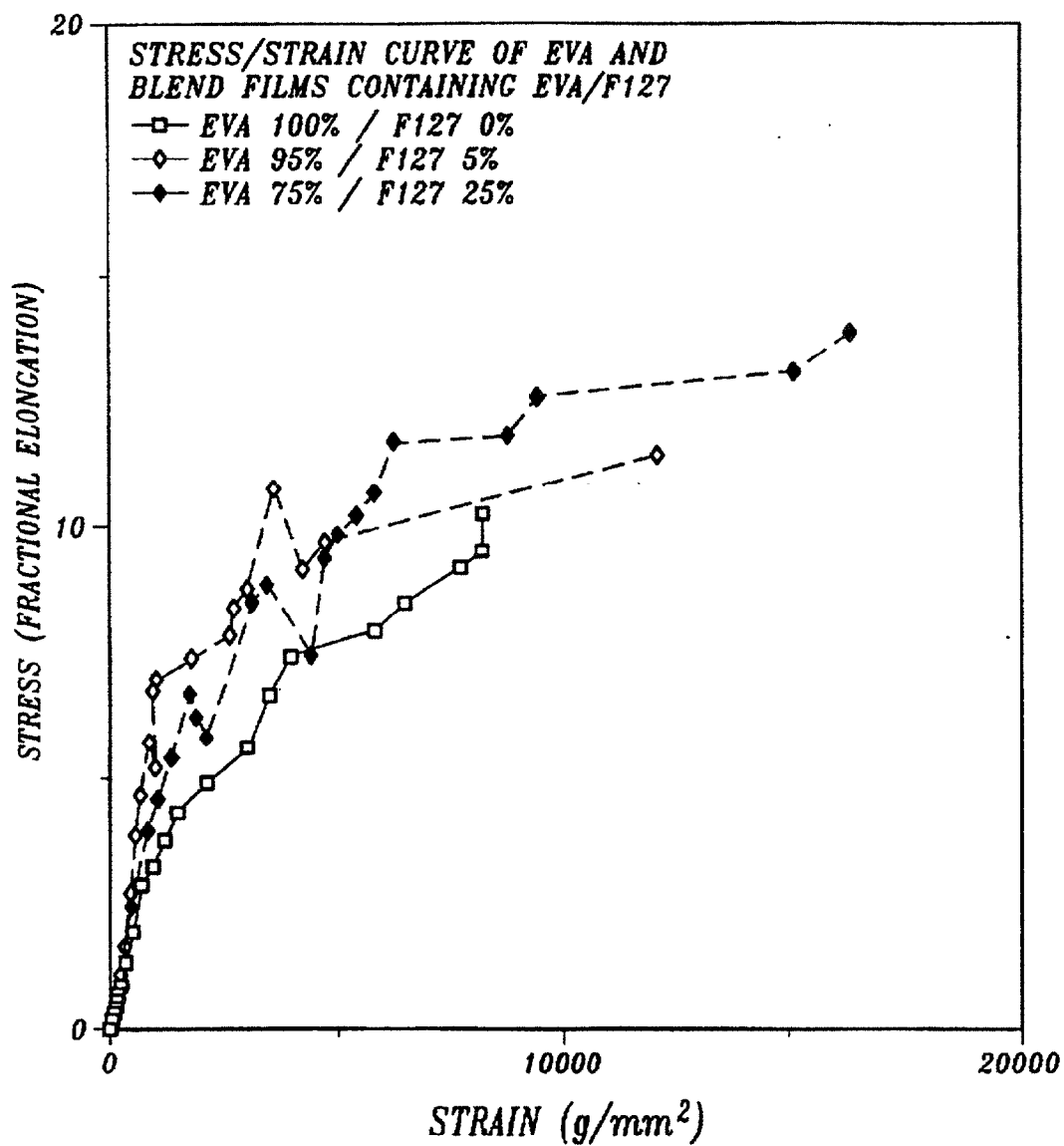
Fig. 16C

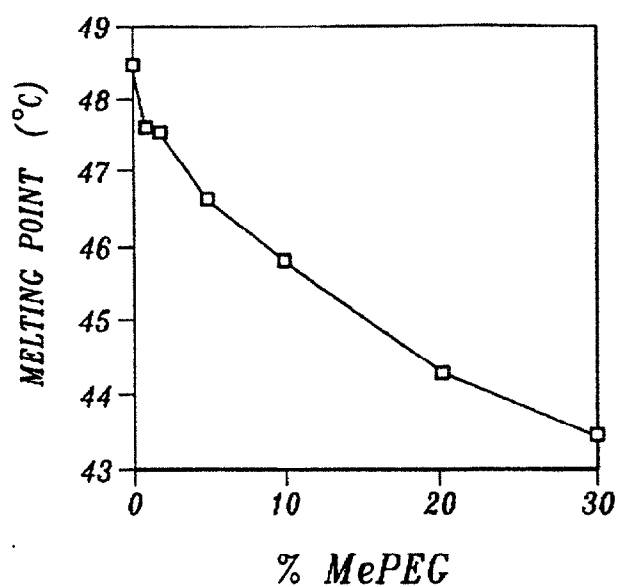
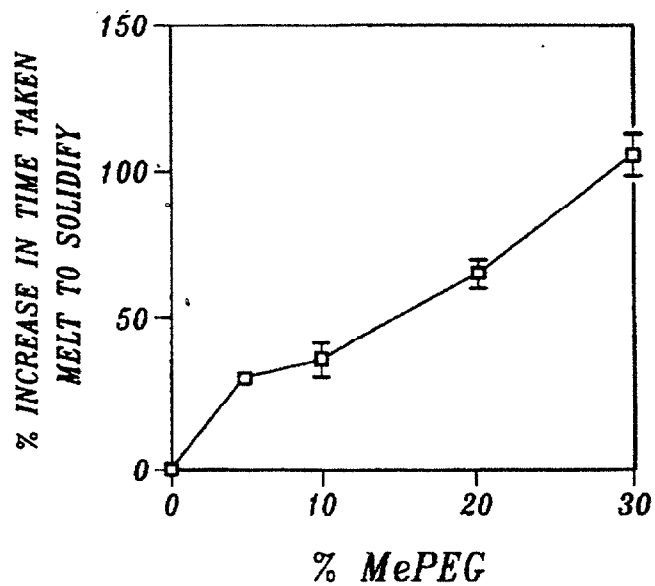
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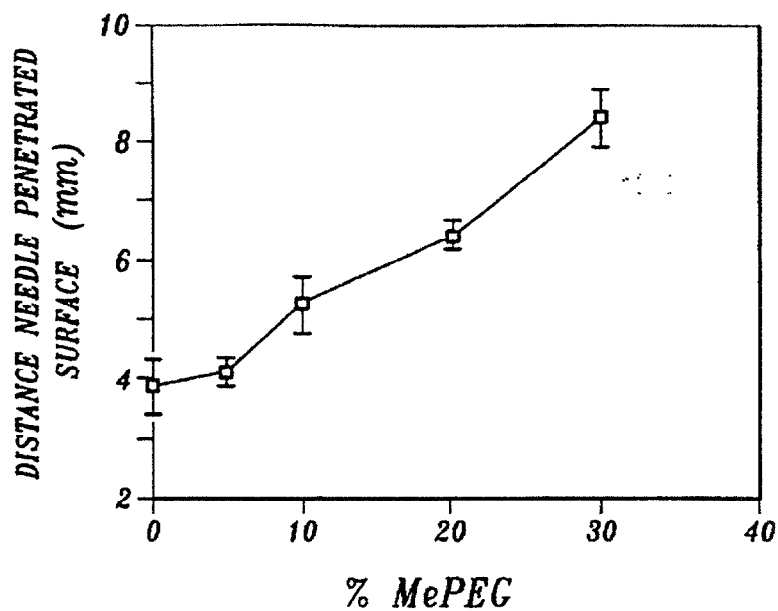
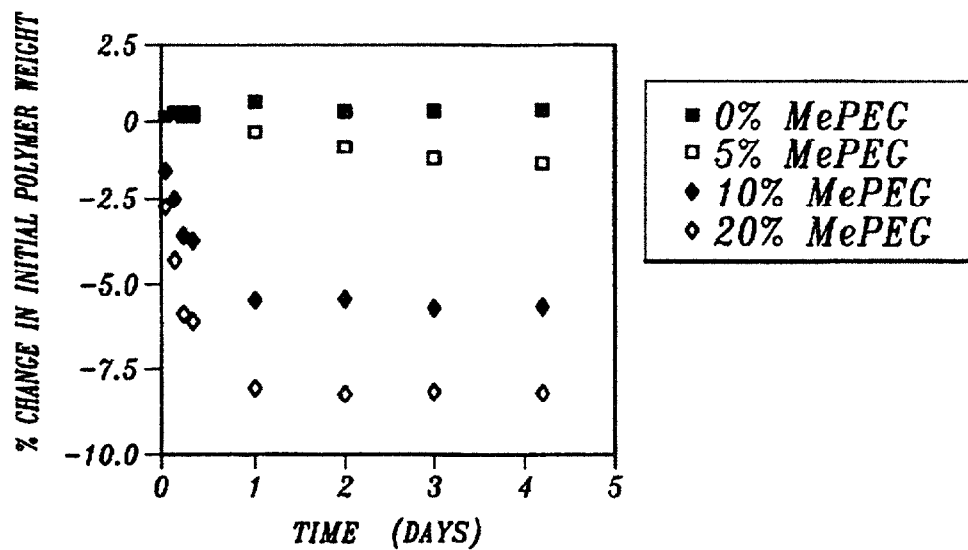
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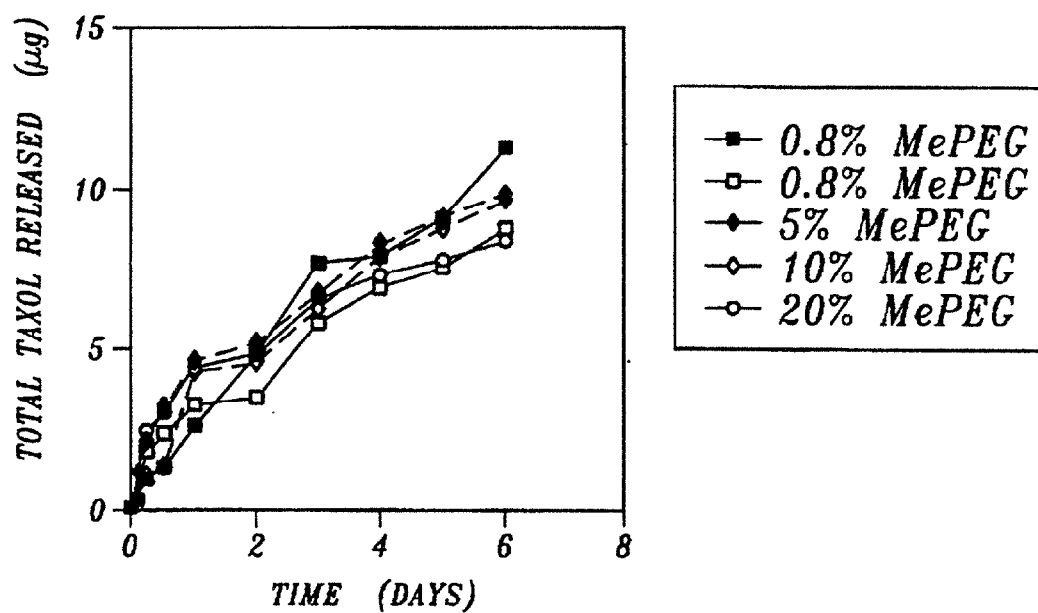
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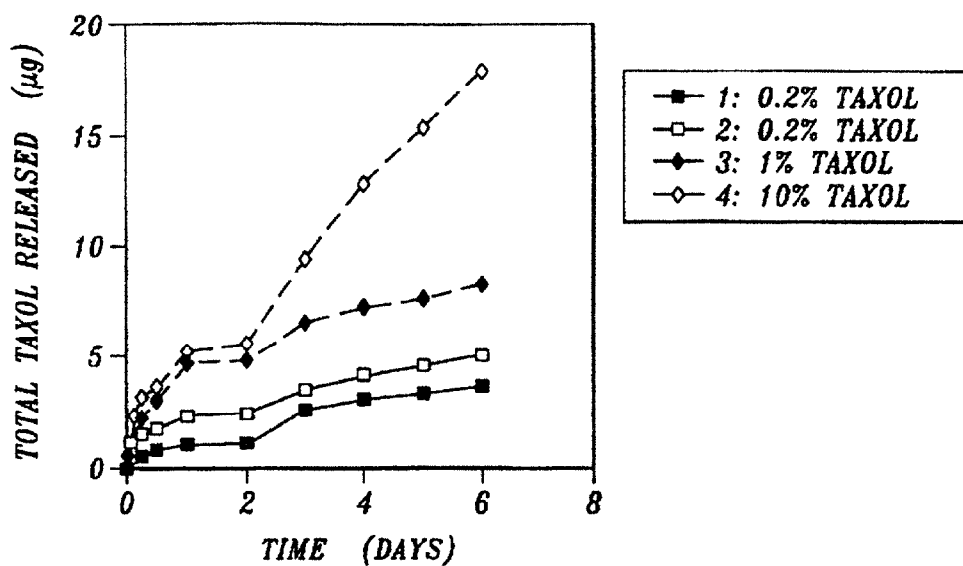
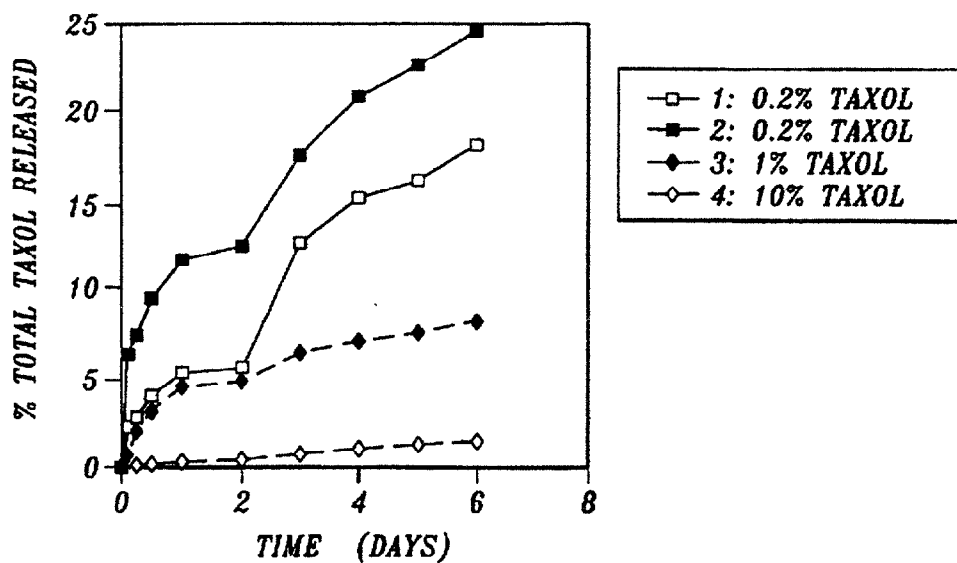
*Fig. 17D*

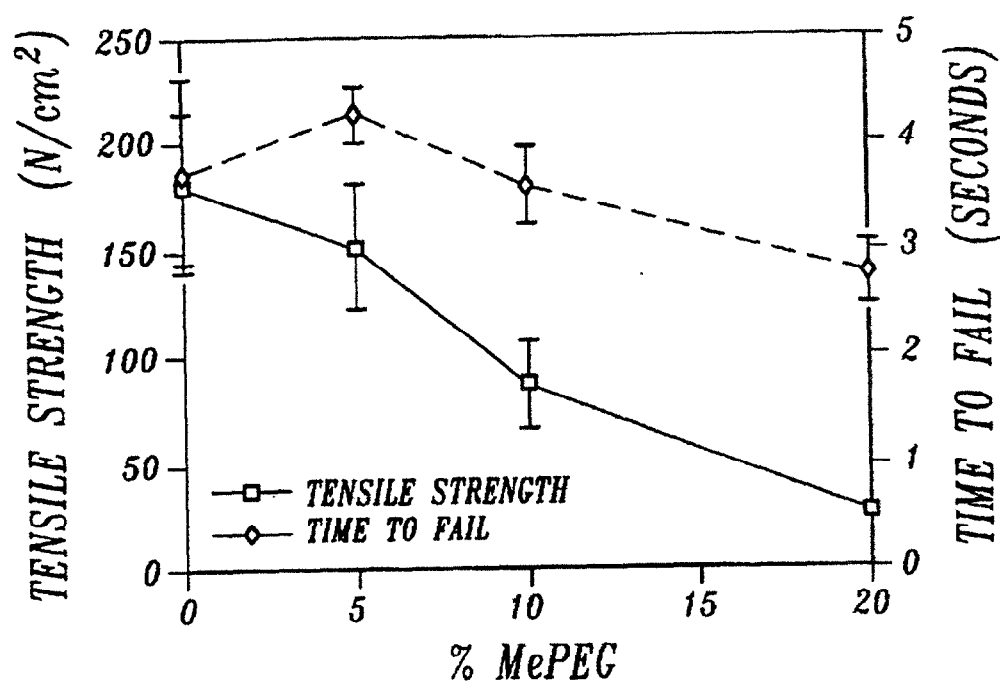
*Fig. 17E*

*Fig. 18A**Fig. 18B*

*Fig. 18C**Fig. 18D*

*Fig. 18E*

*Fig. 18F**Fig. 18G*

*Fig. 18H*

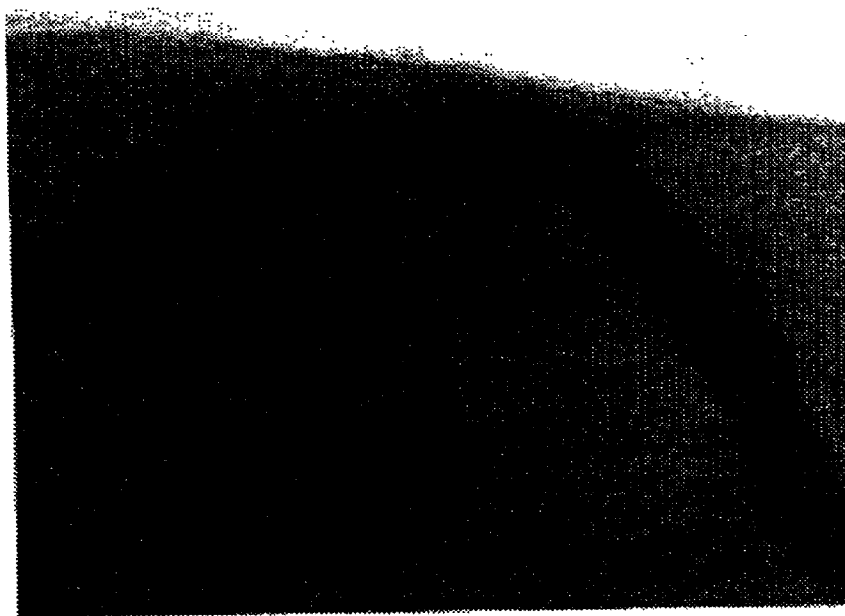


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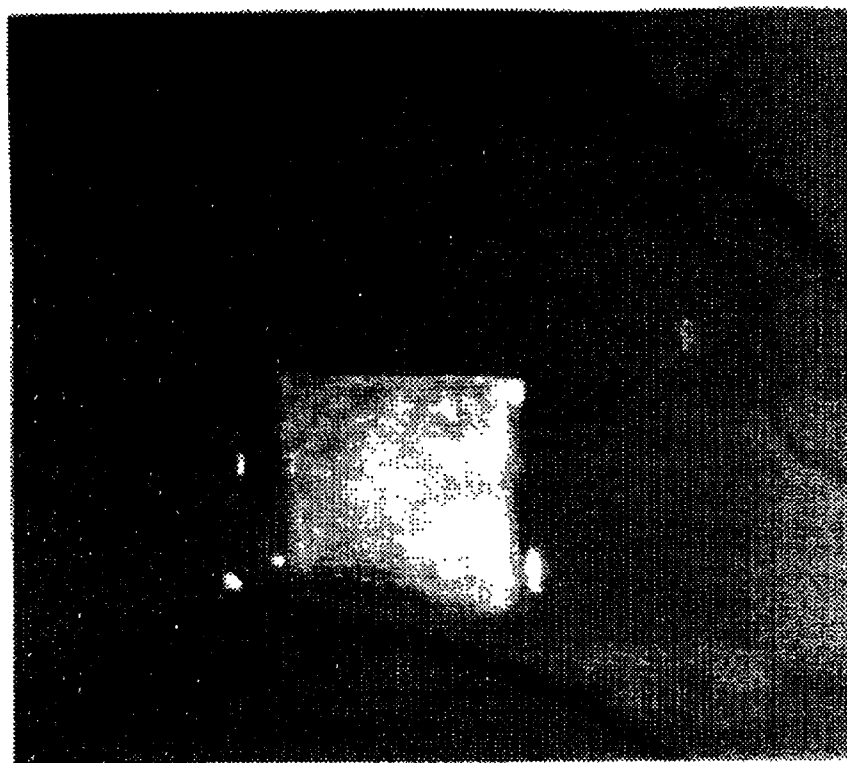


Fig. 19B

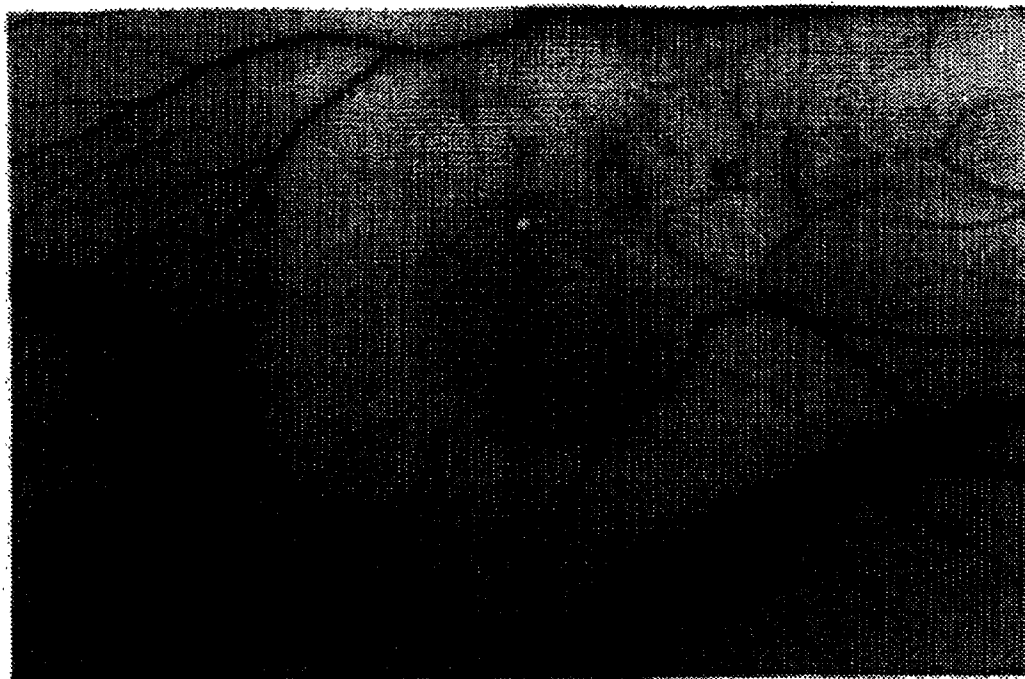


Fig. 19C



Fig. 19D



Fig. 20A



Fig. 20B

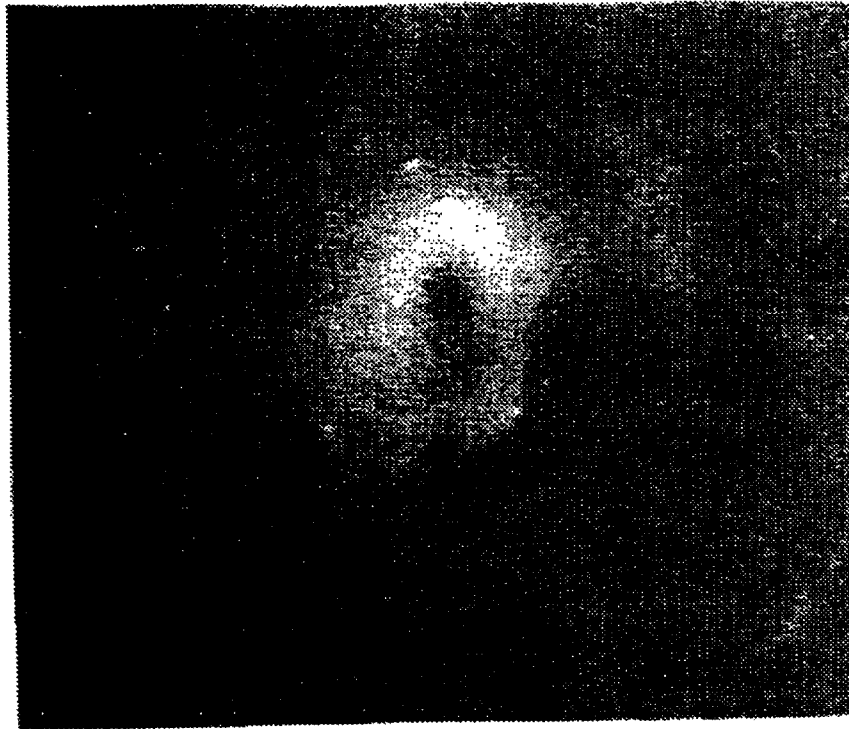


Fig. 20C

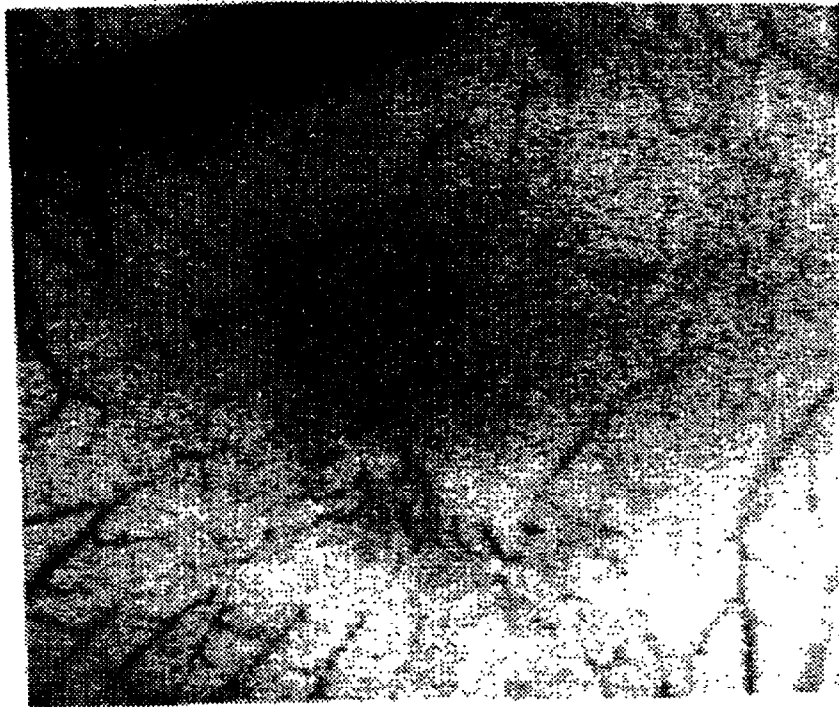
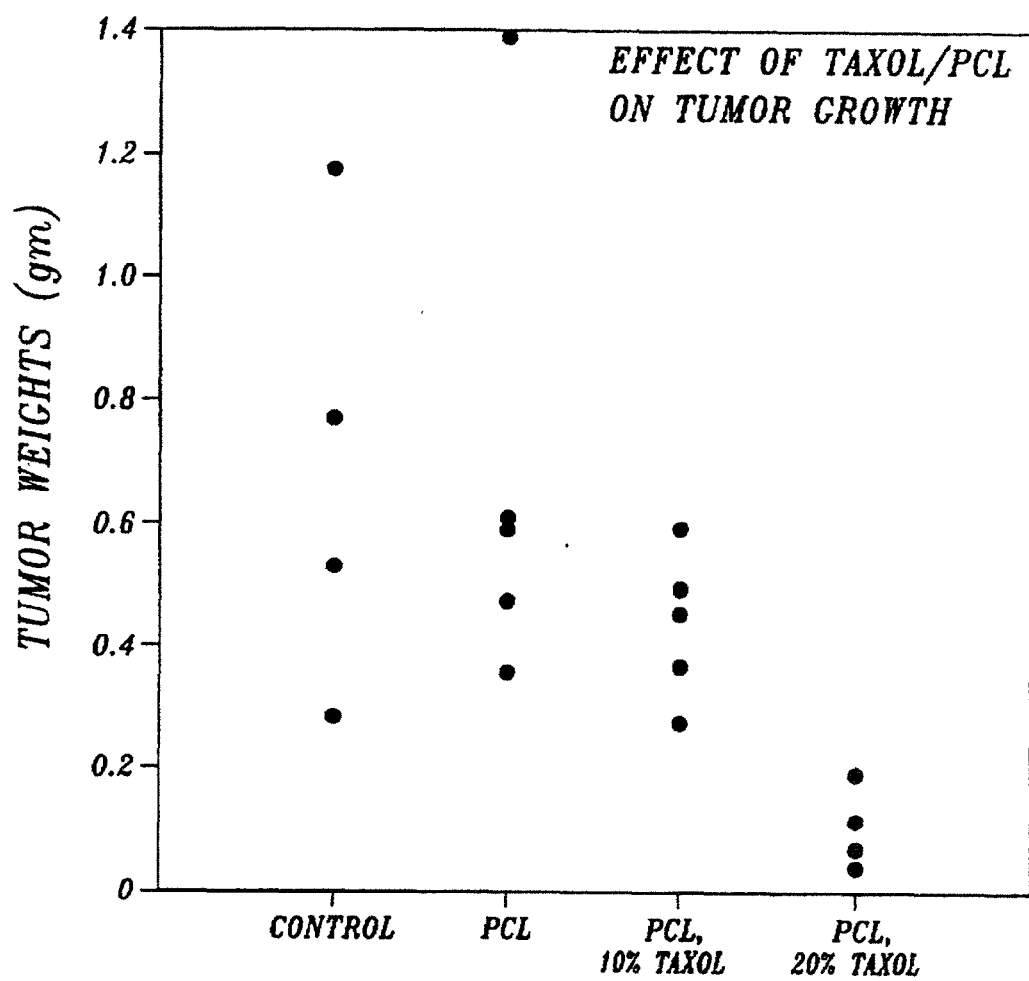


Fig. 20D

*Fig. 21A*

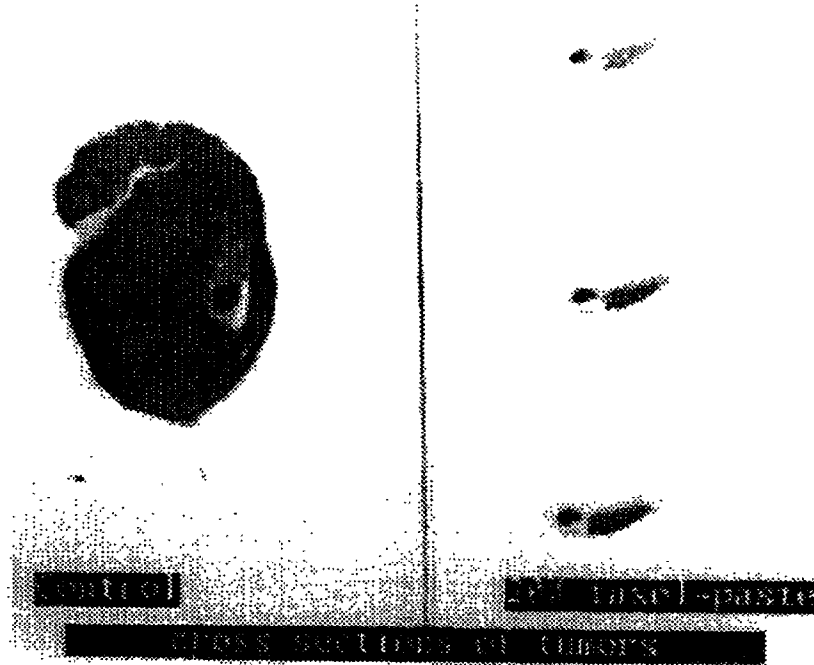


Fig. 21B

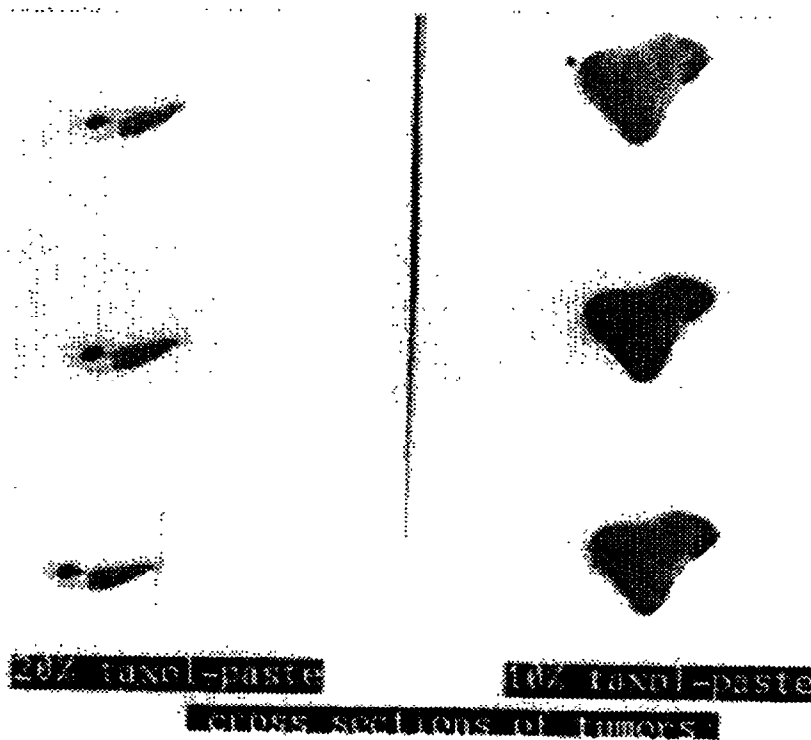


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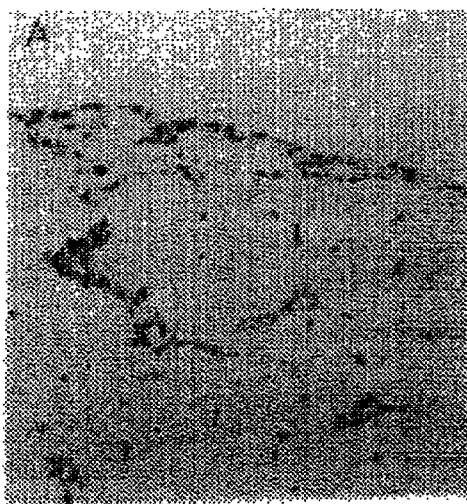


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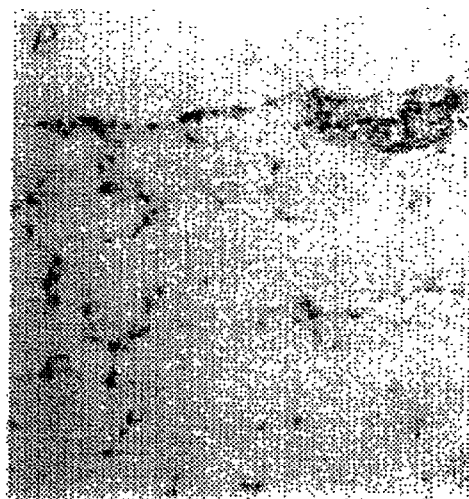


Fig. 22B



Fig. 22C

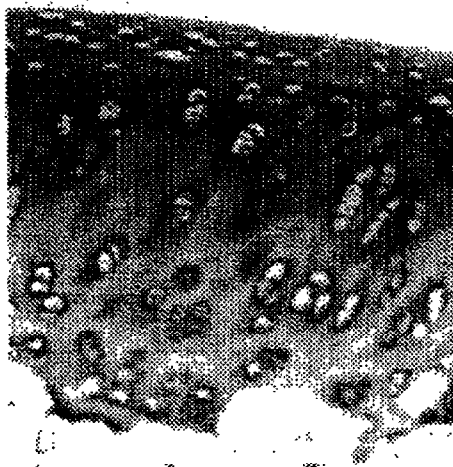


Fig. 22D



Fig. 23A

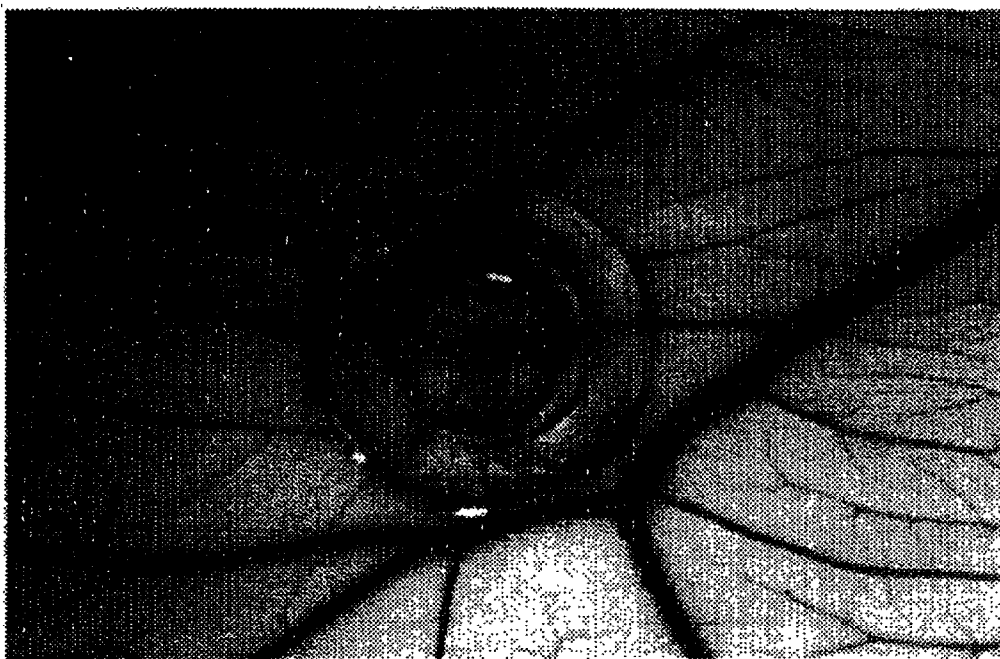


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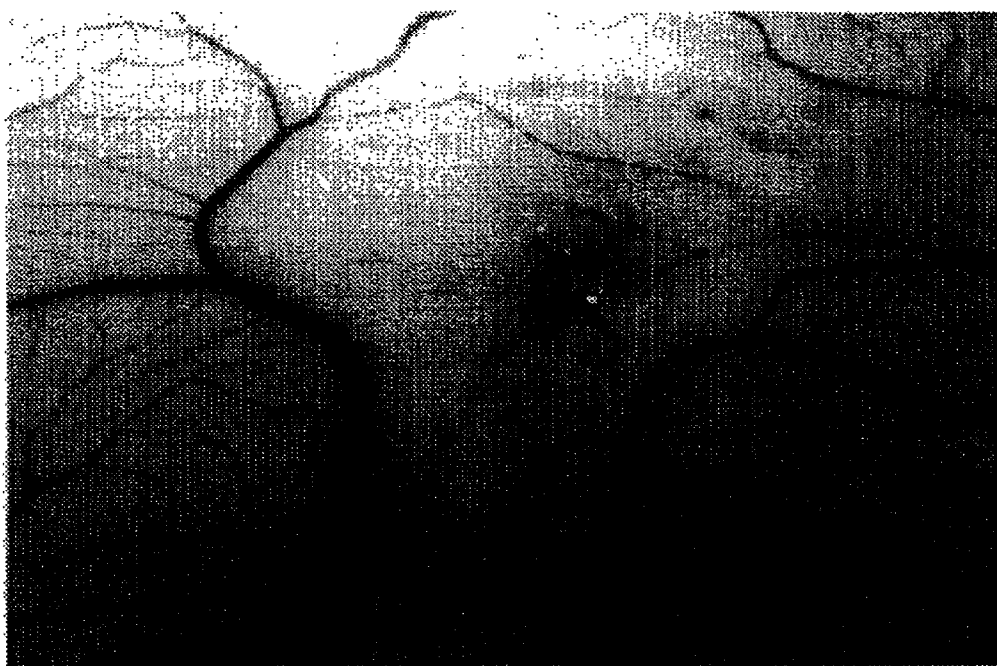


Fig. 24A



Fig. 24B



Fig. 25



Fig. 26

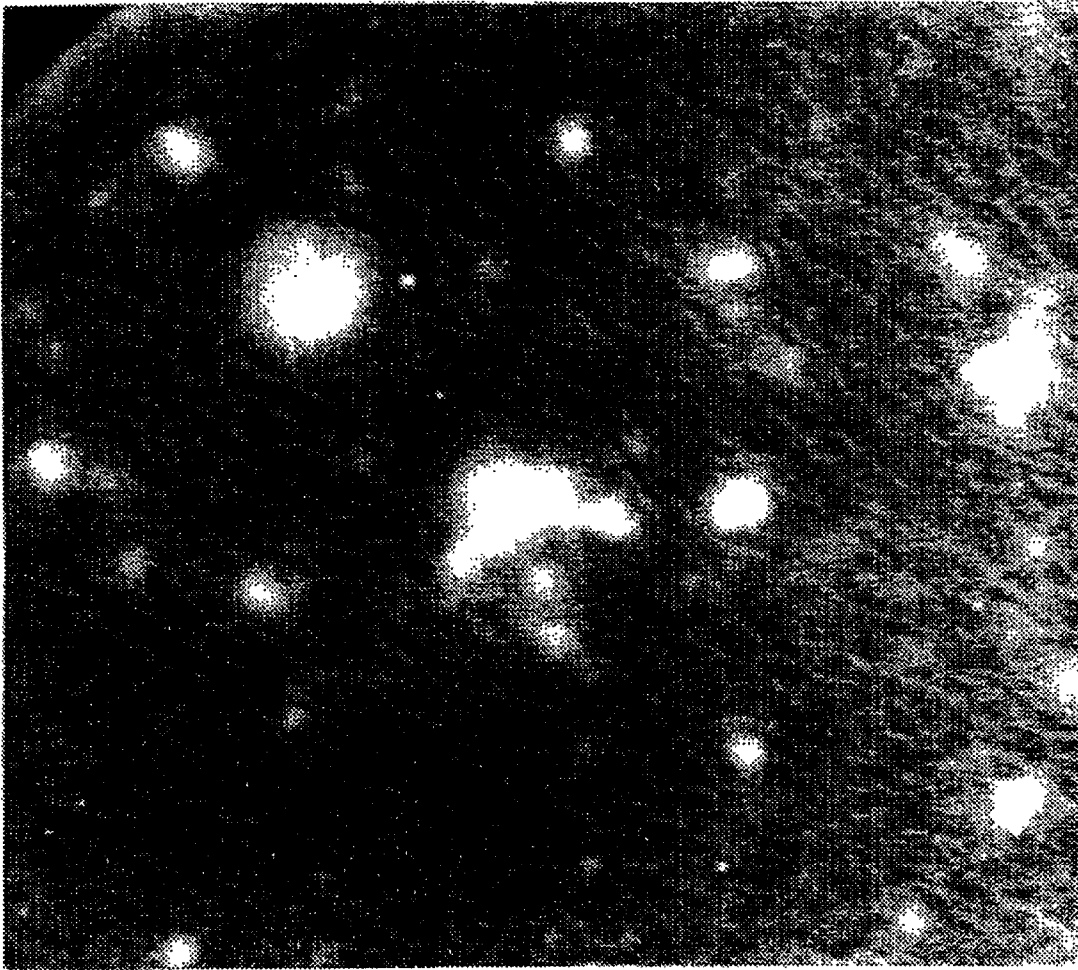


Fig. 27

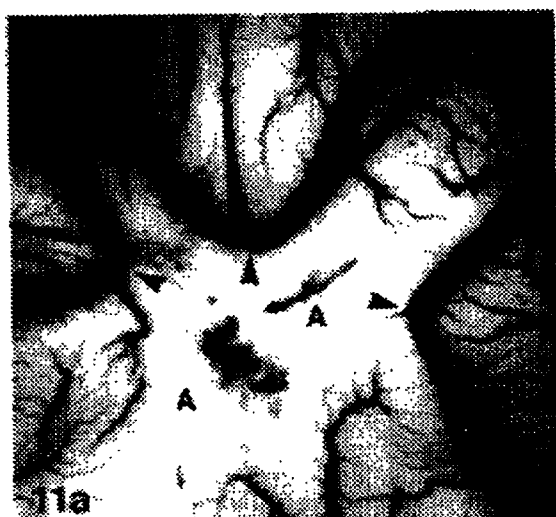


Fig. 28A

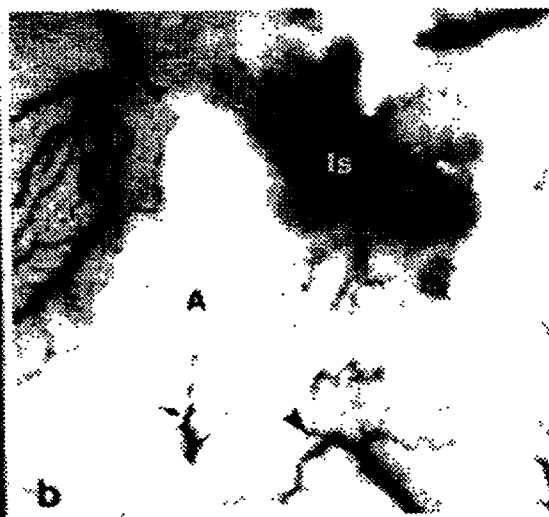
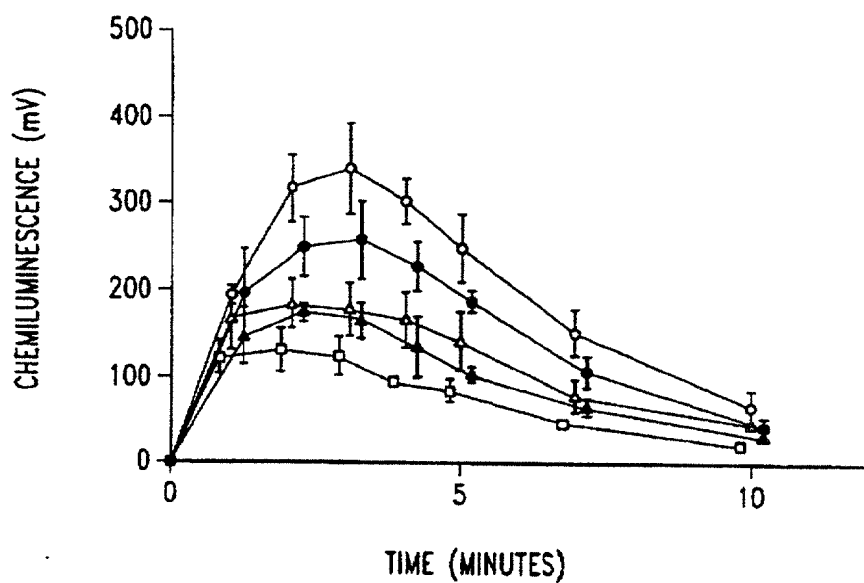
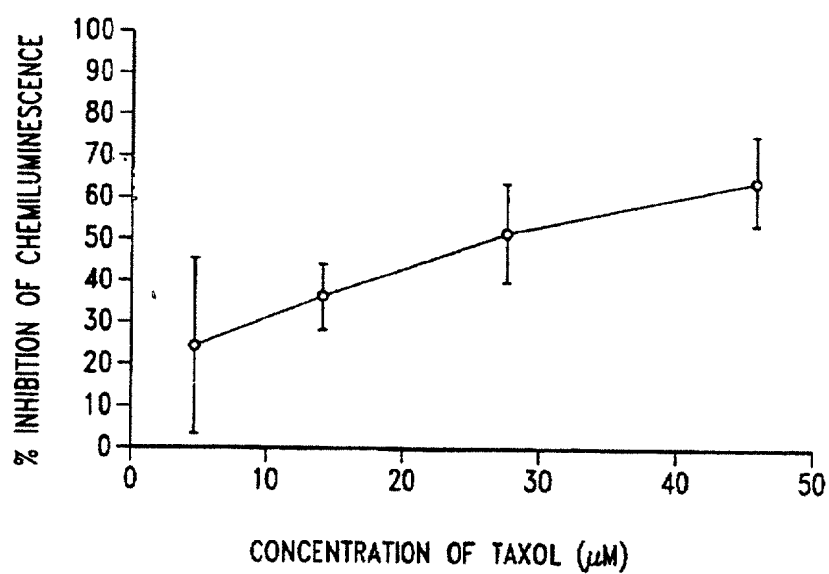
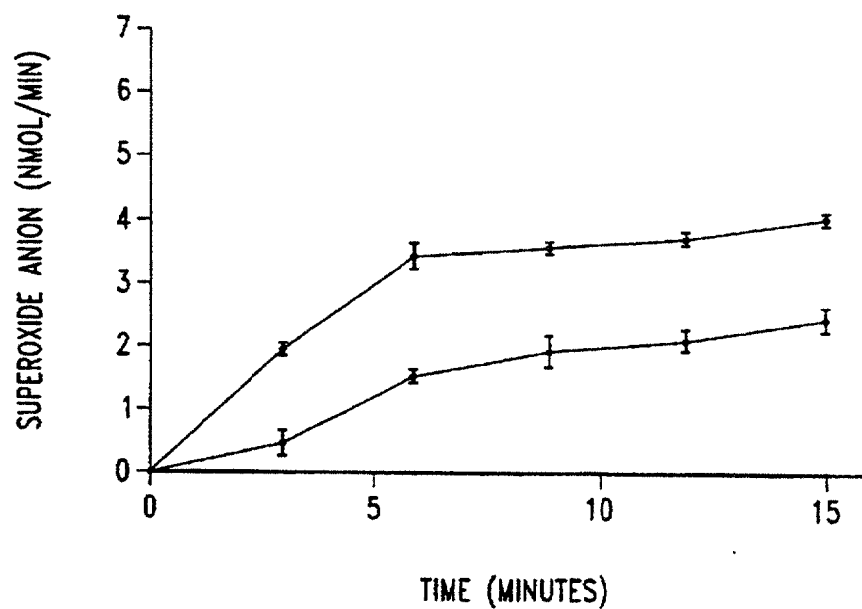
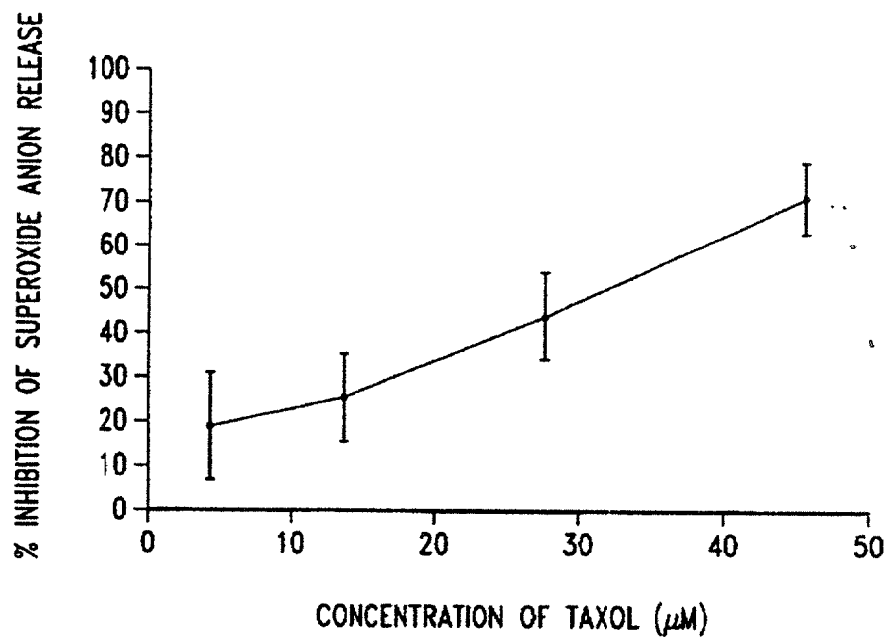
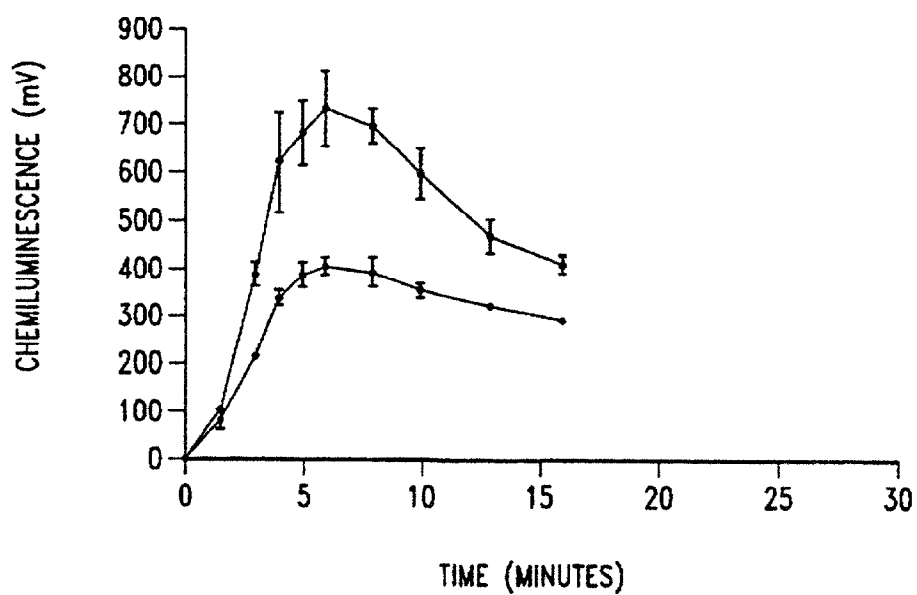
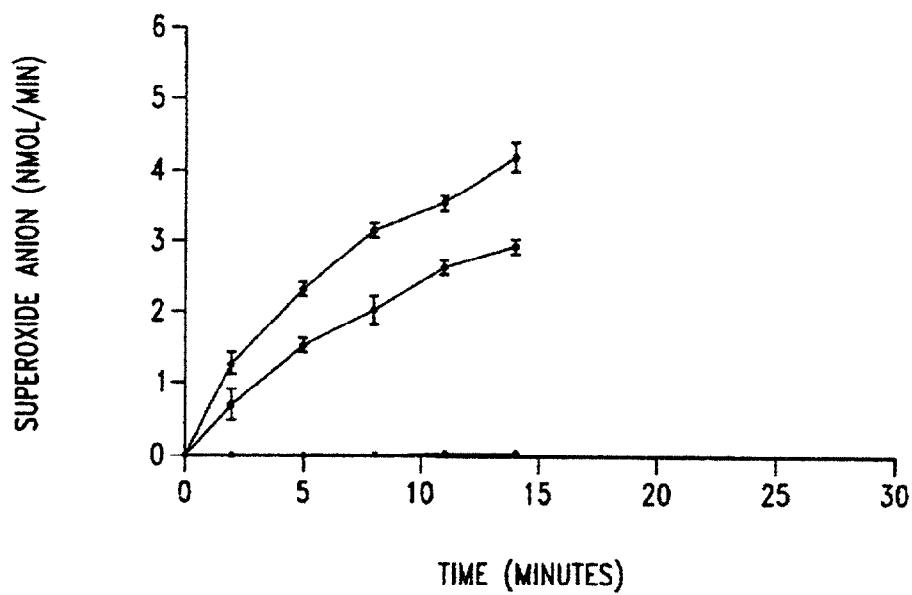
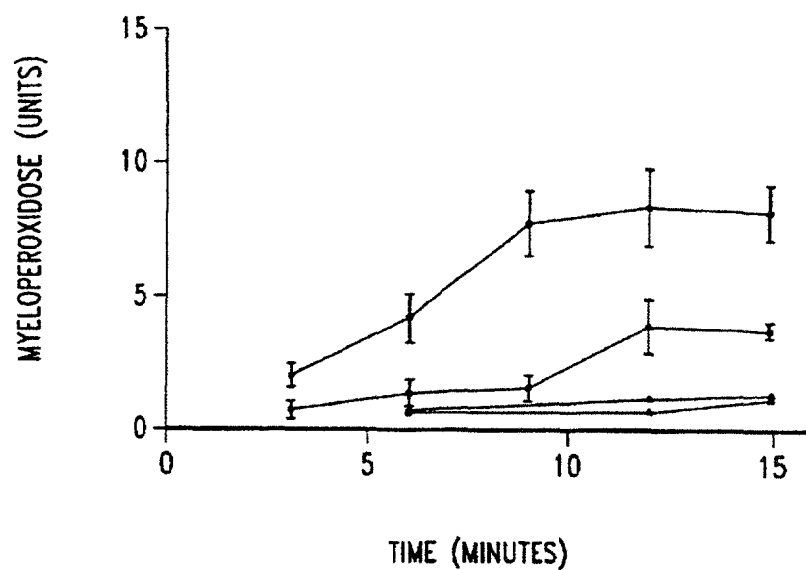
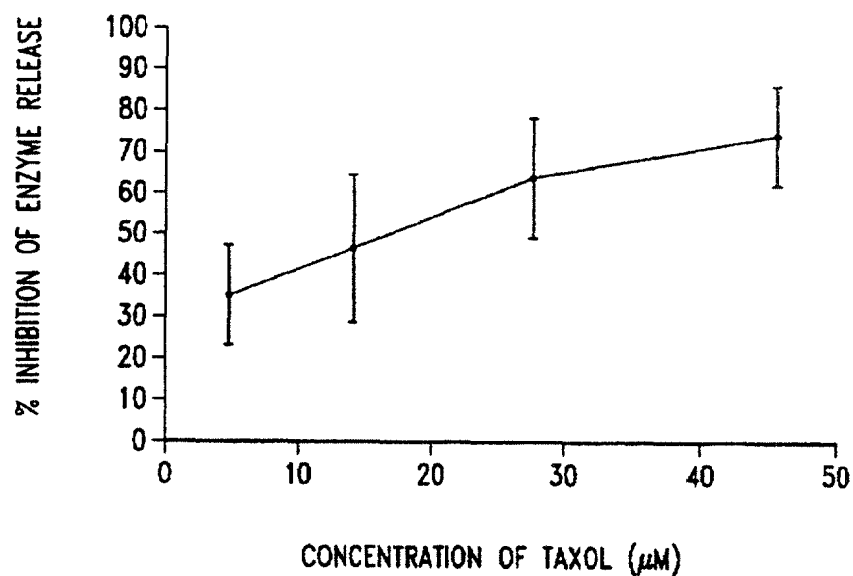


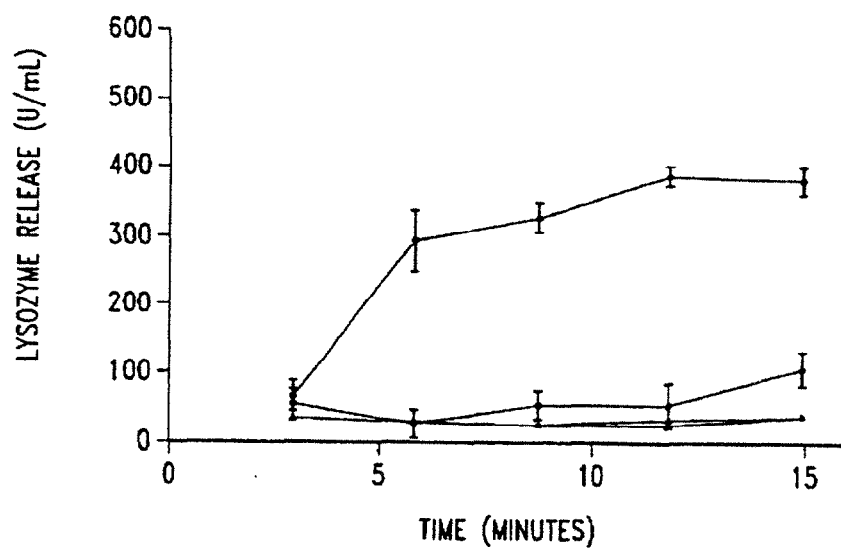
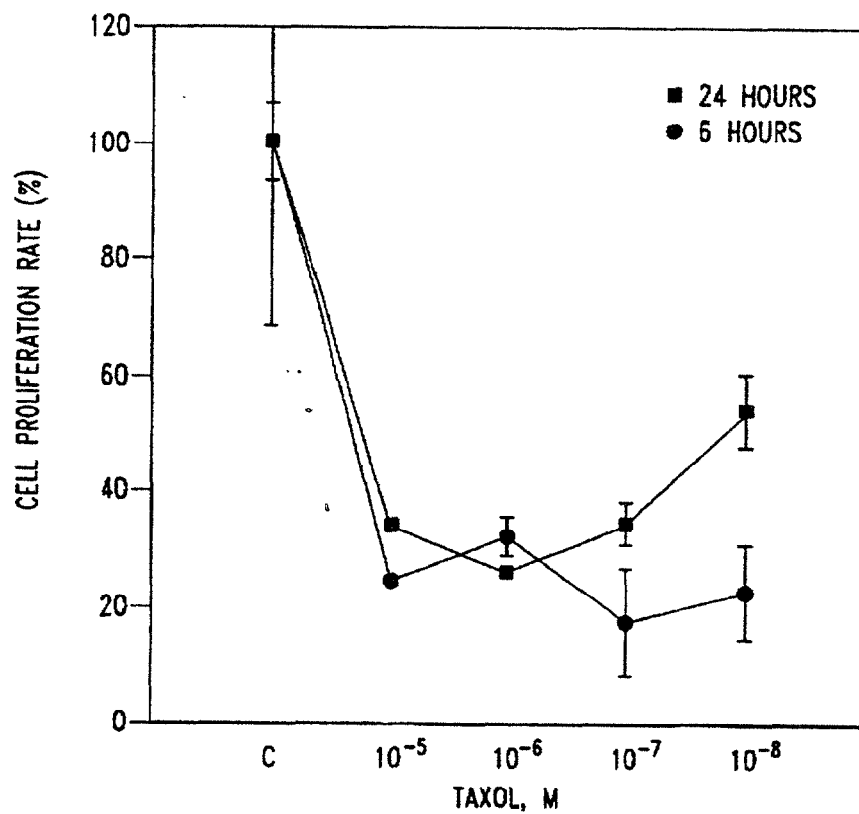
Fig. 28B

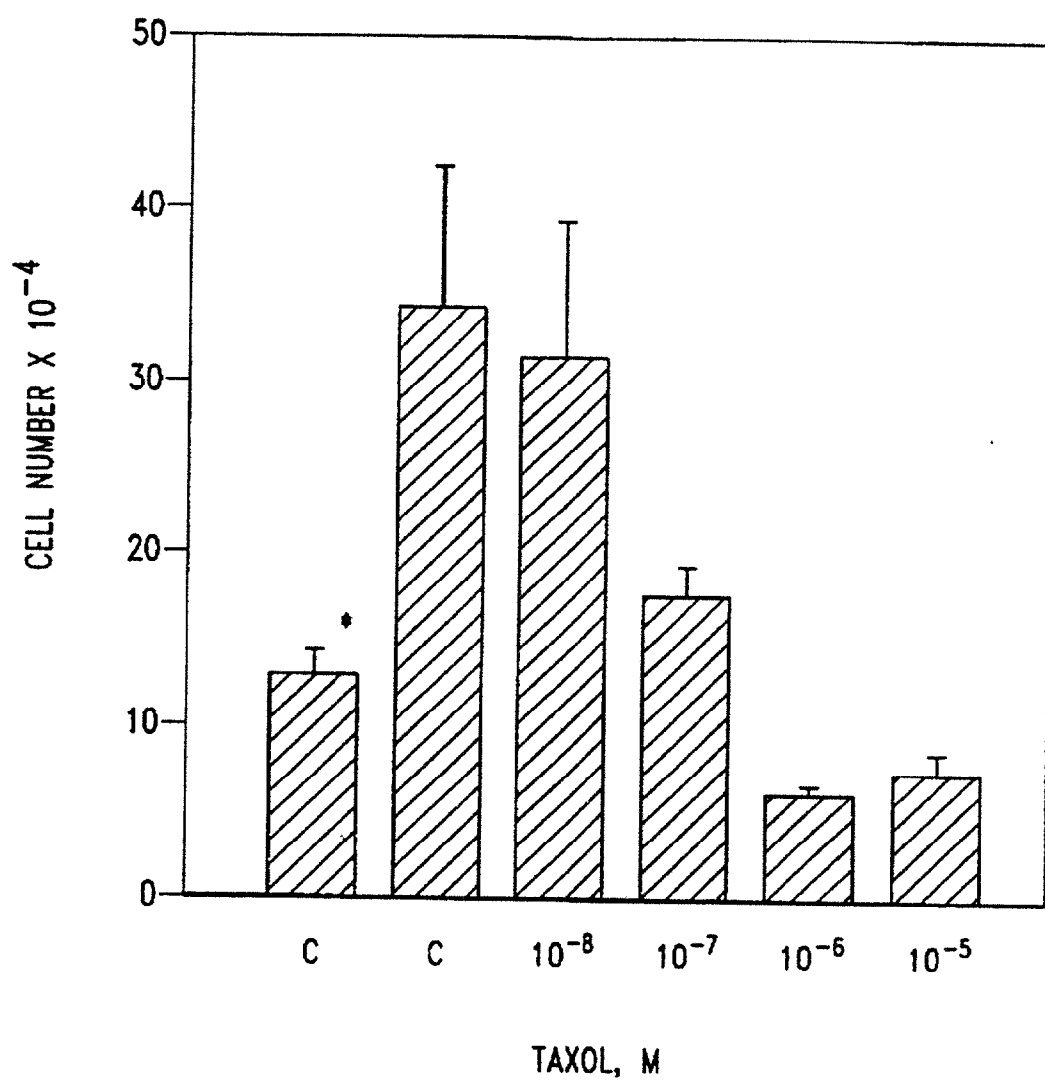
*Fig. 29A**Fig. 29B*

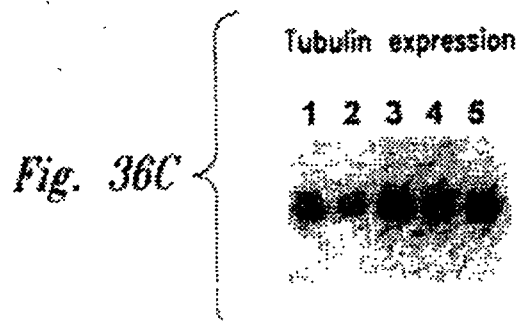
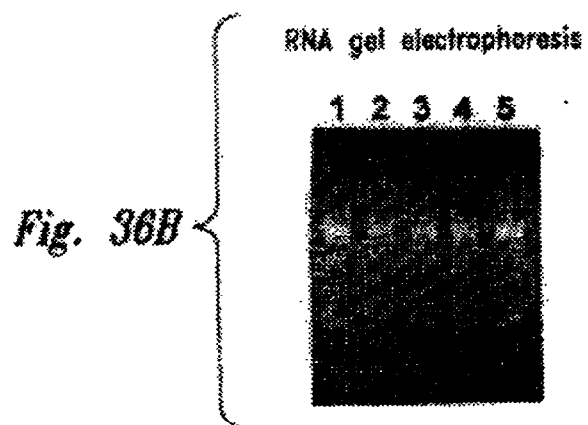
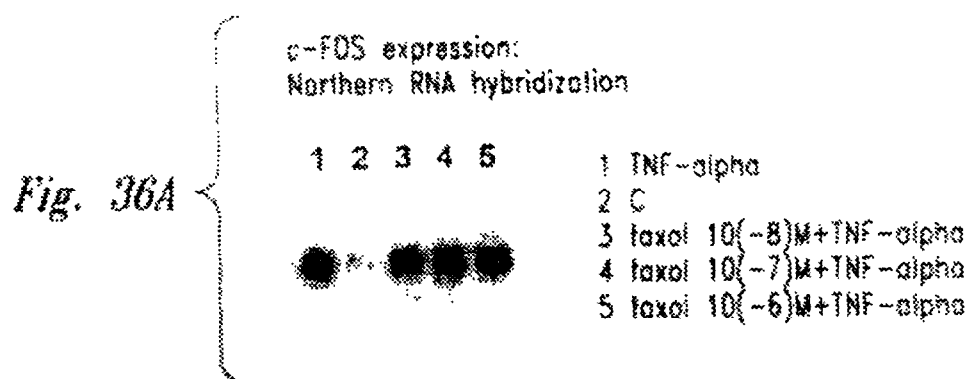
*Fig. 30A**Fig. 30B*

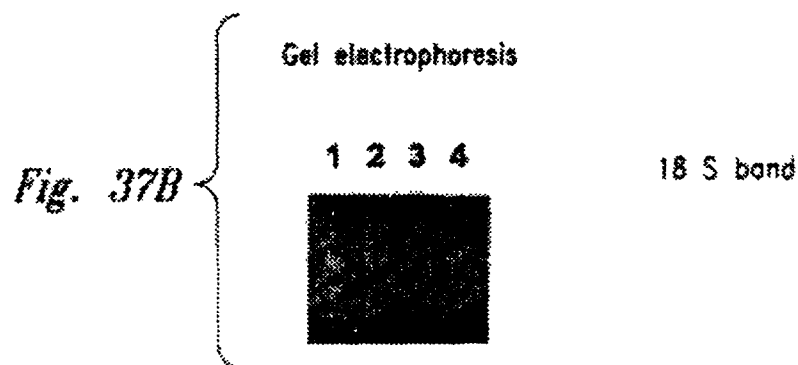
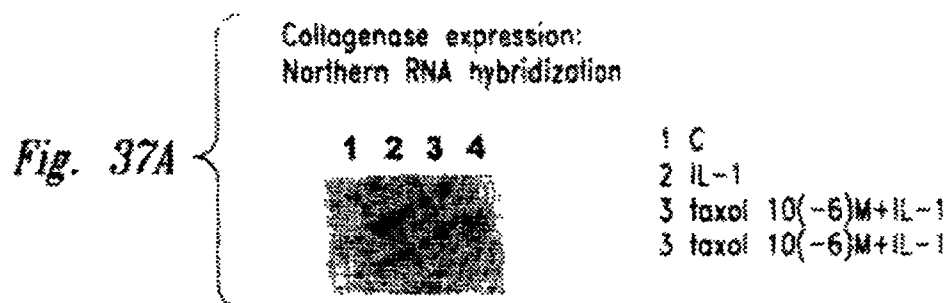
*Fig. 31A**Fig. 31B*

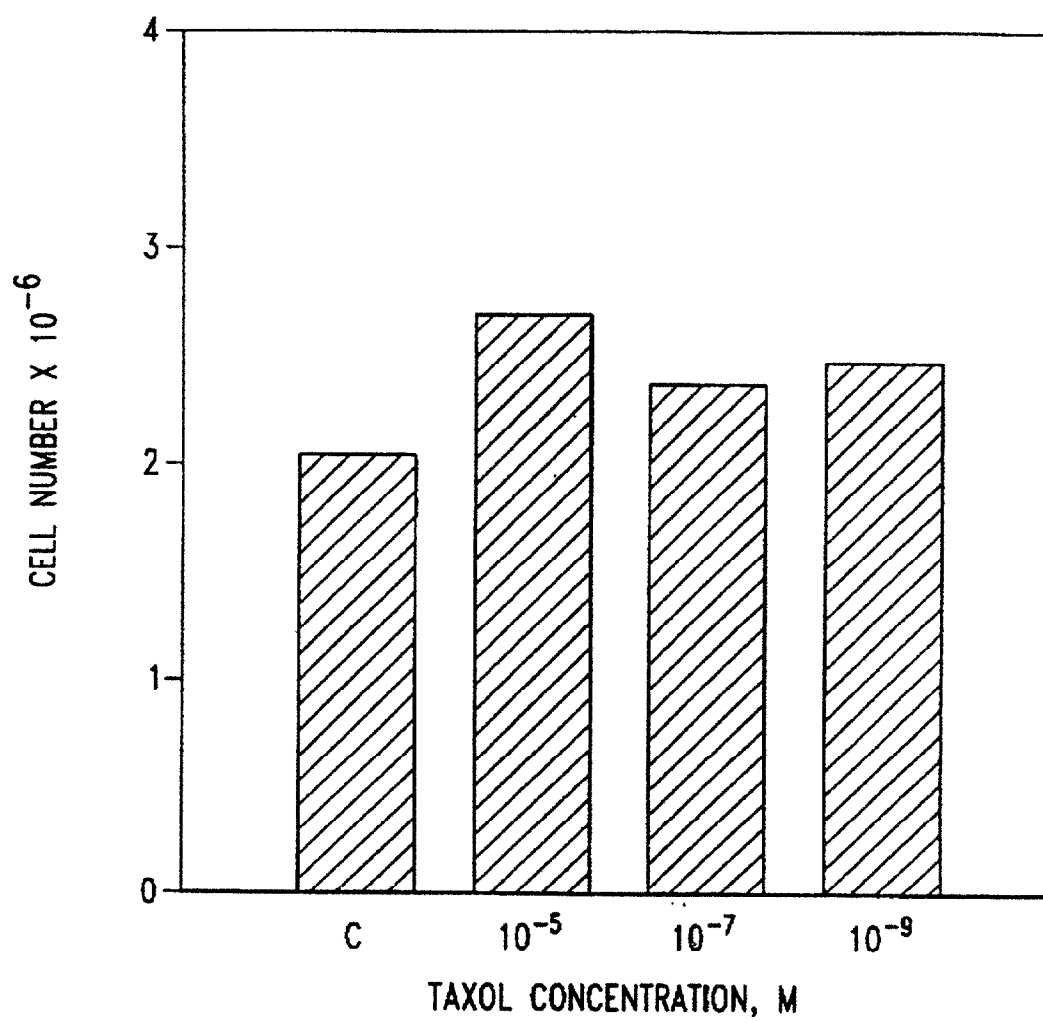
*Fig. 32A**Fig. 32B*

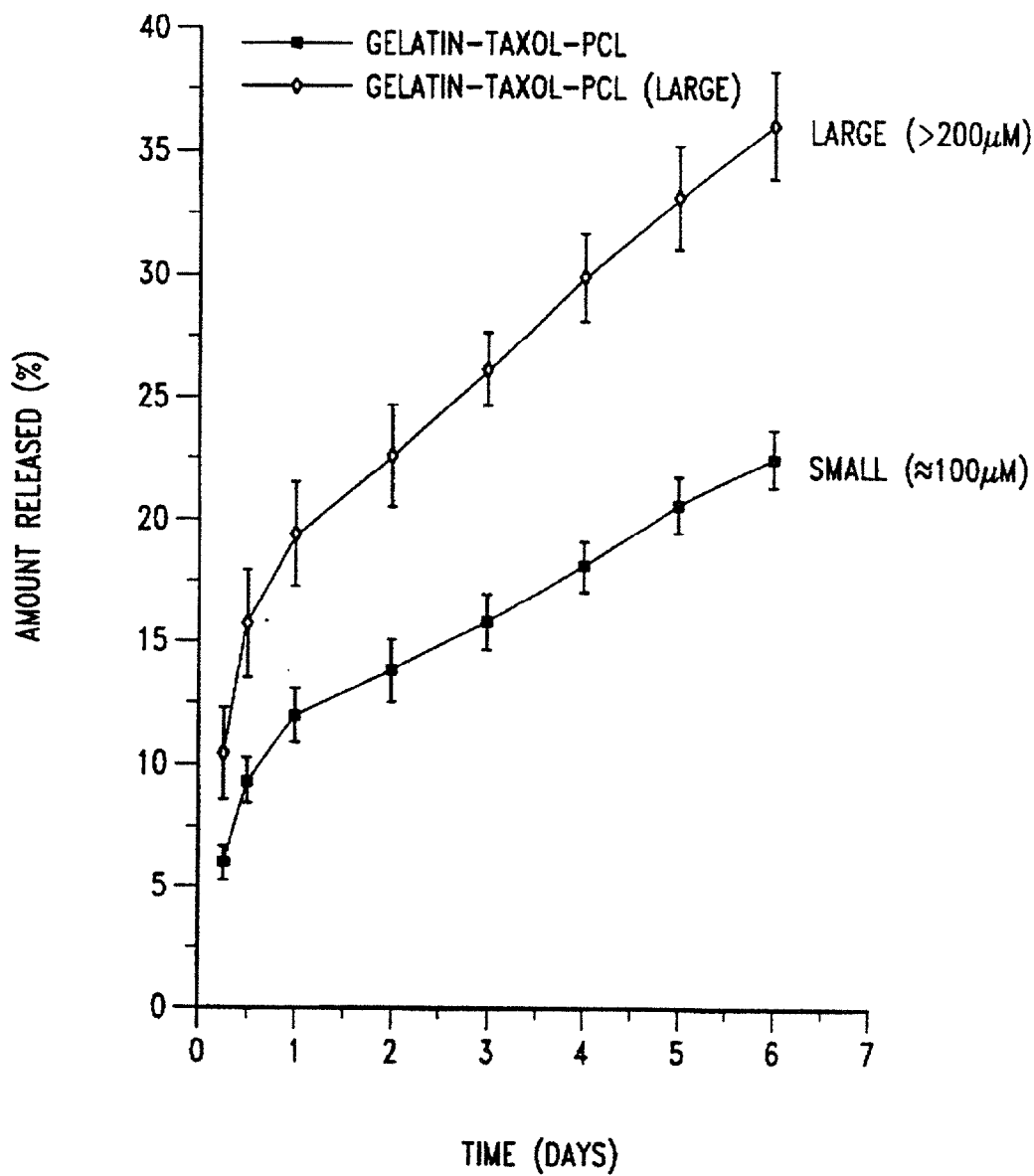
*Fig. 33**Fig. 34*

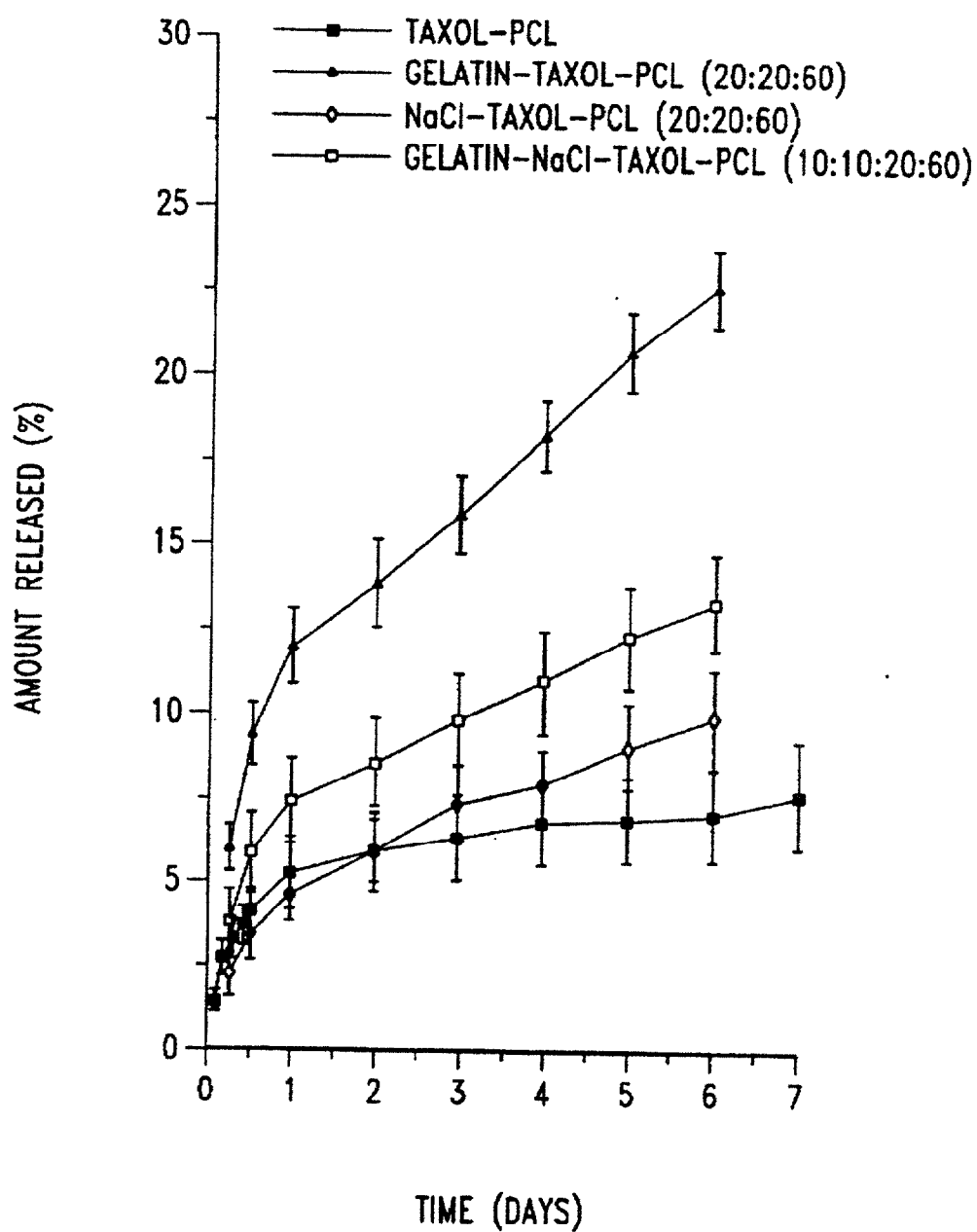
*Fig. 35*

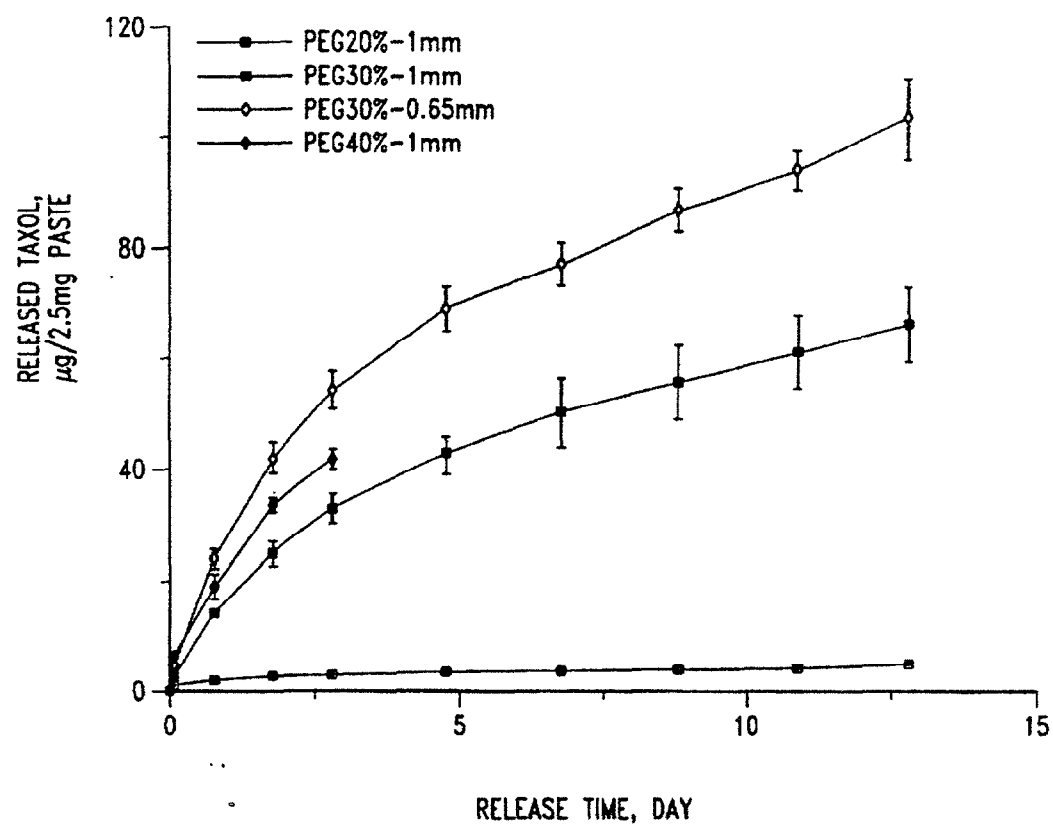




*Fig. 38*

*Fig. 39*

*Fig. 40*

*Fig. 41*

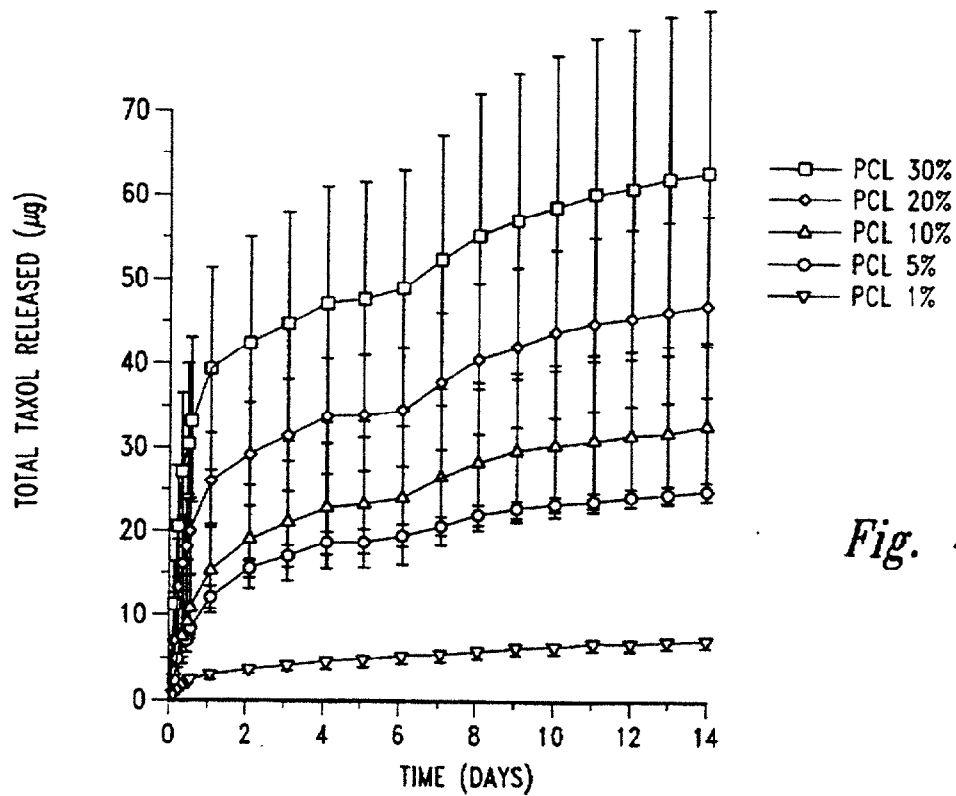


Fig. 42A

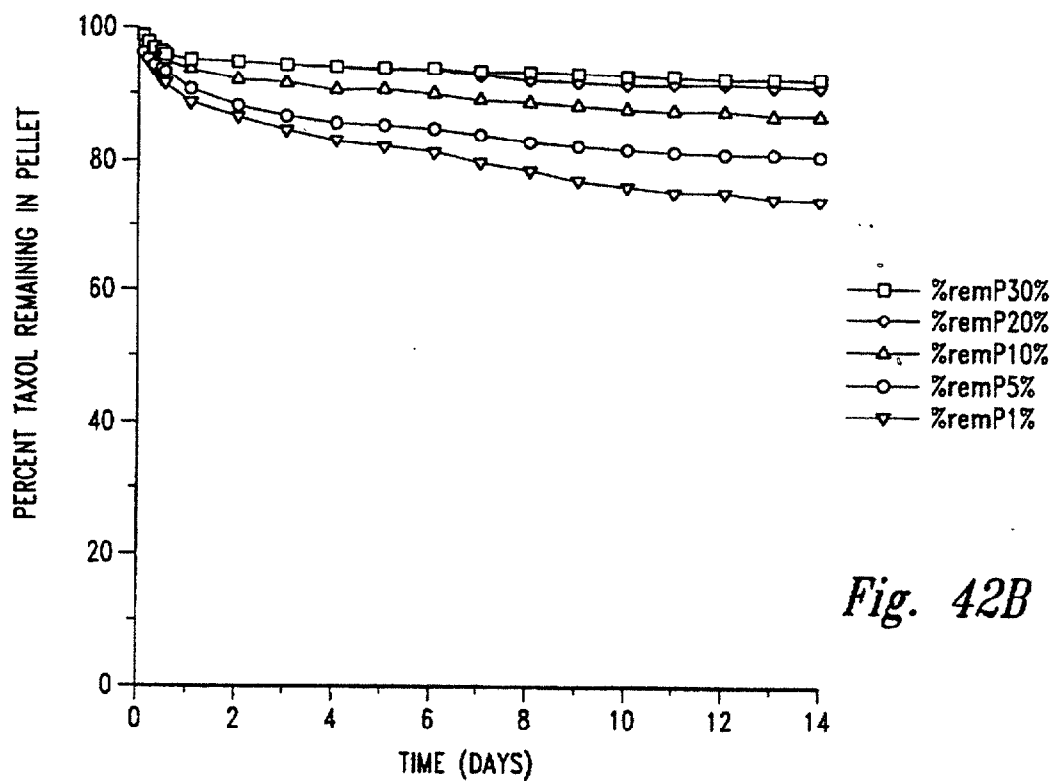
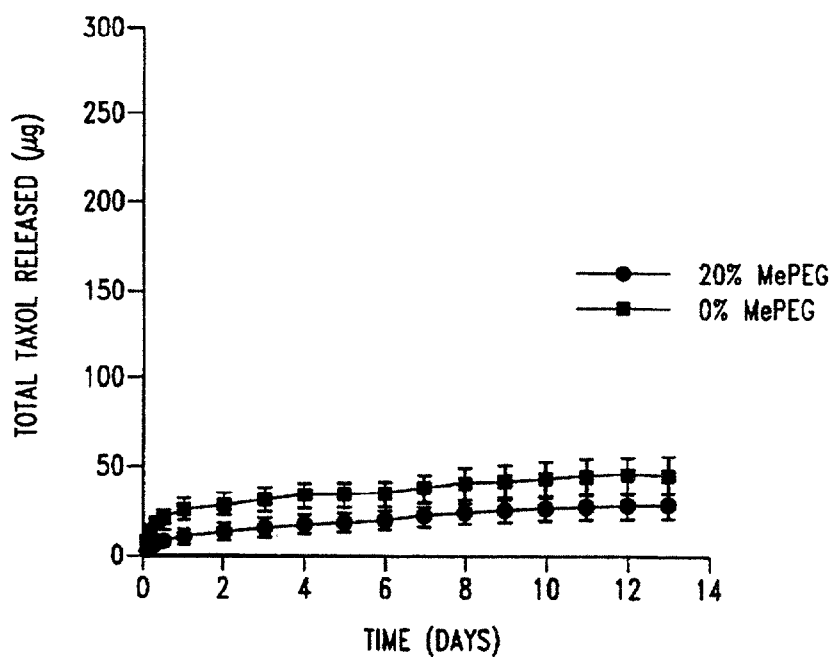
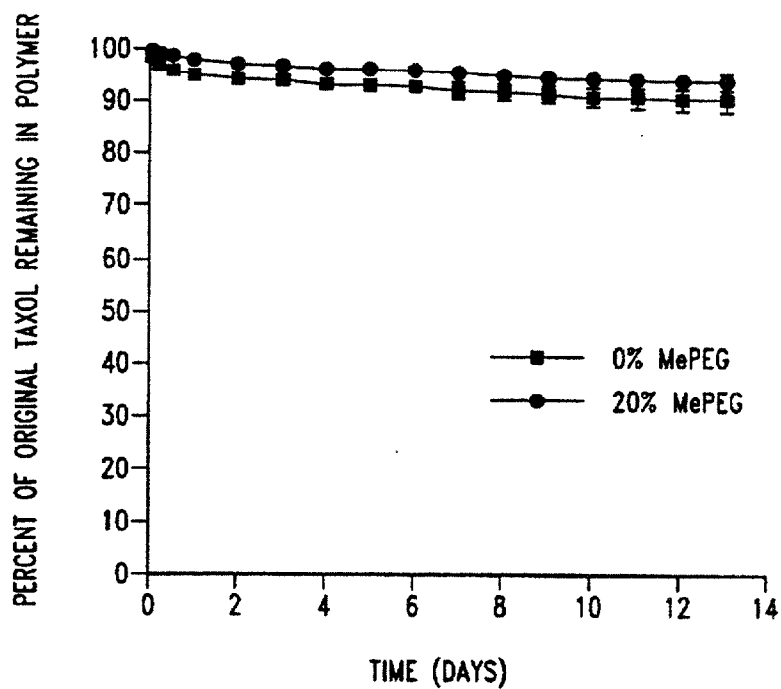
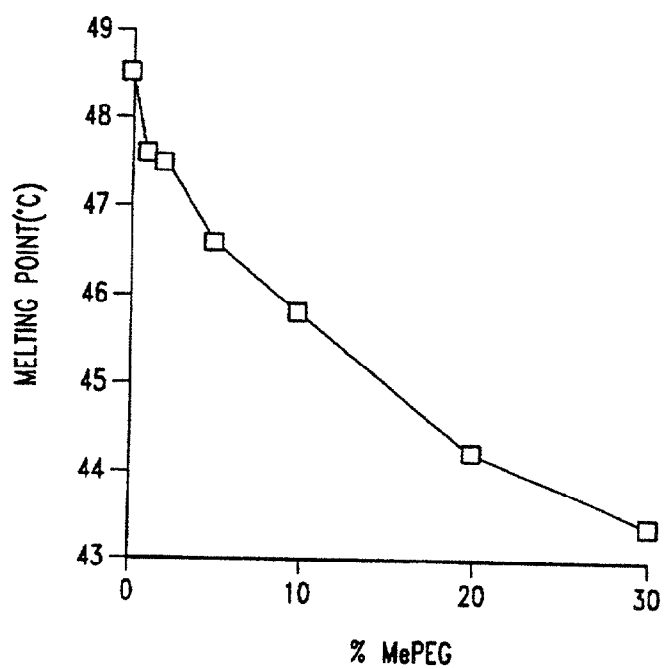
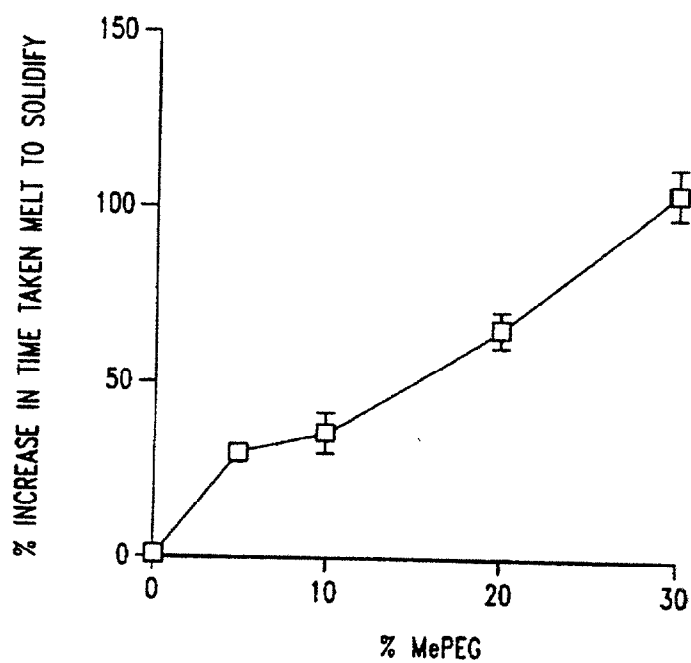
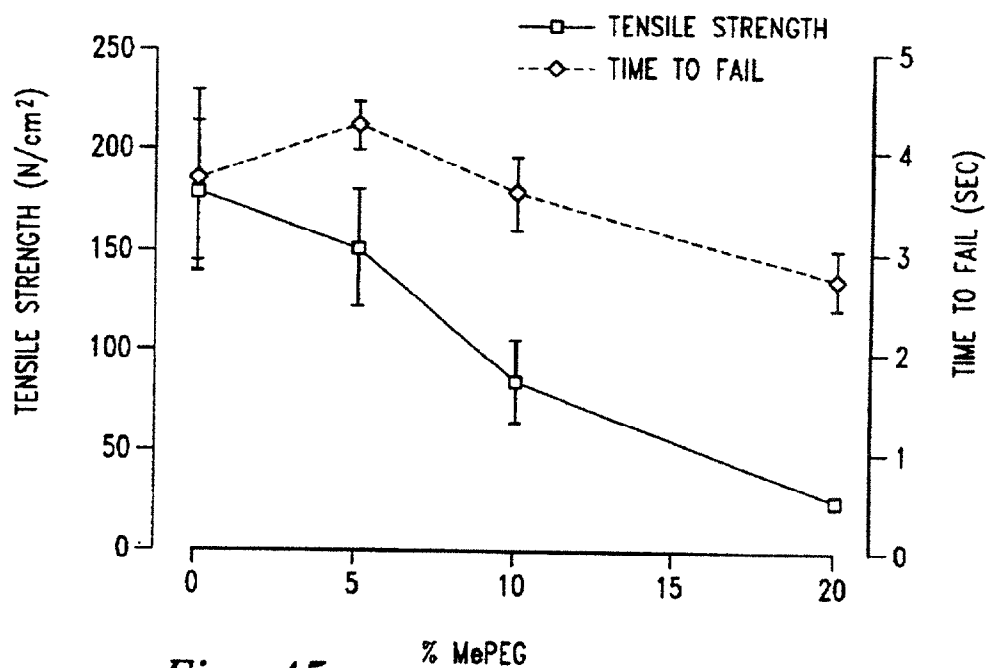
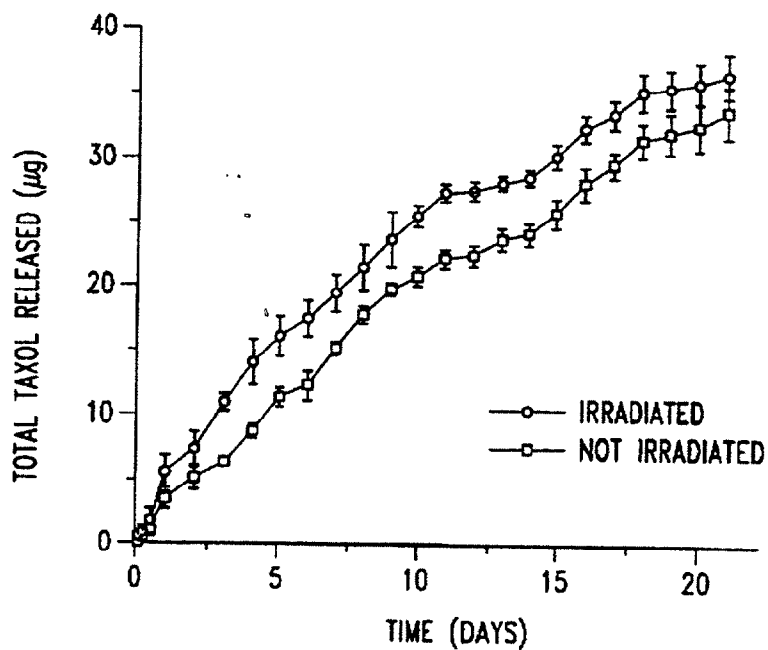
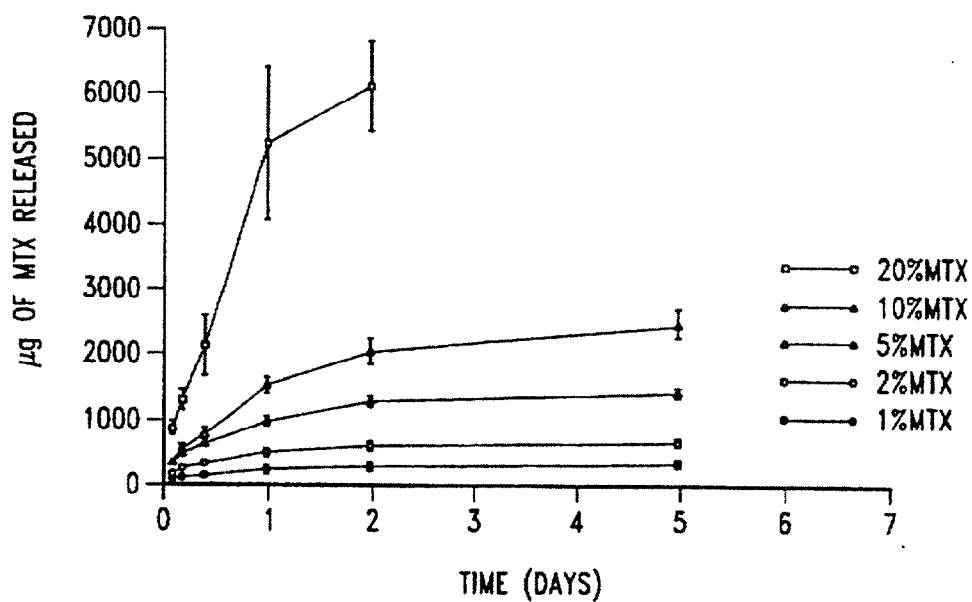
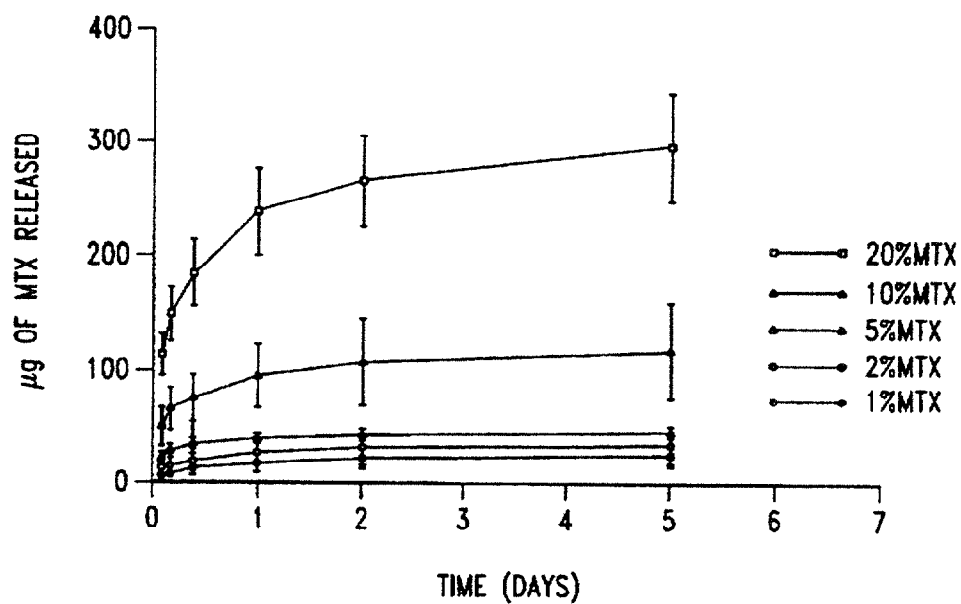


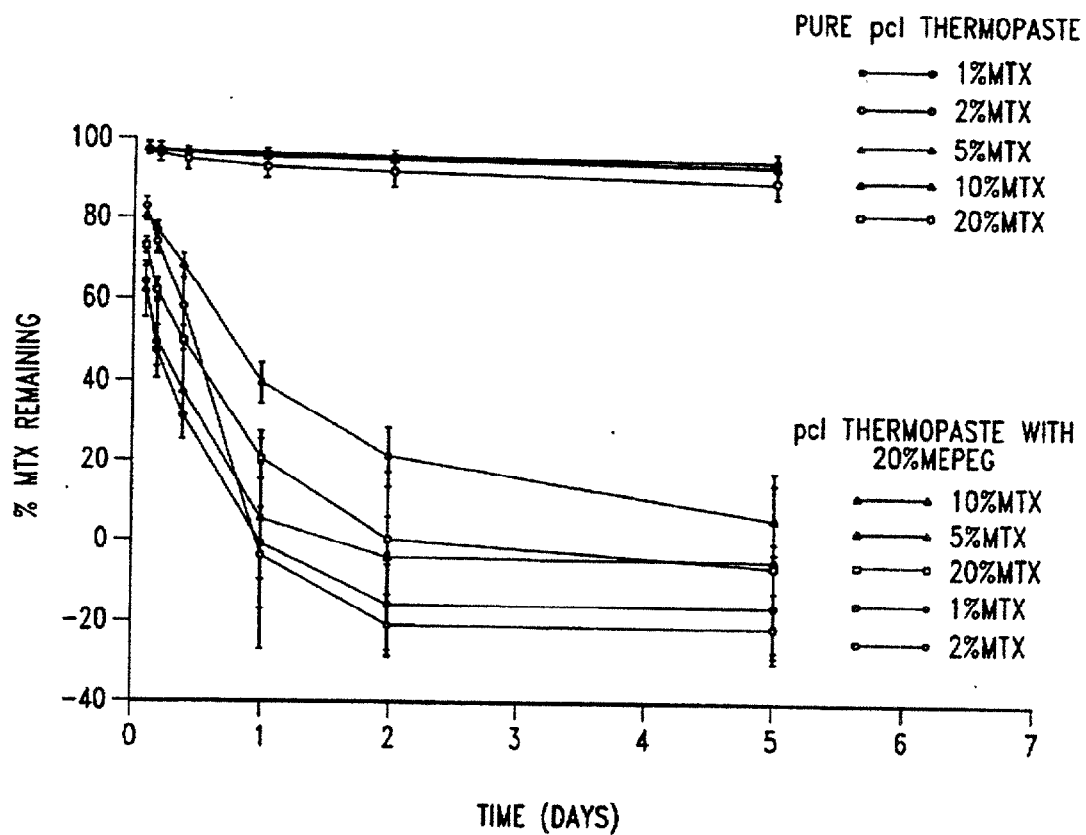
Fig. 42B

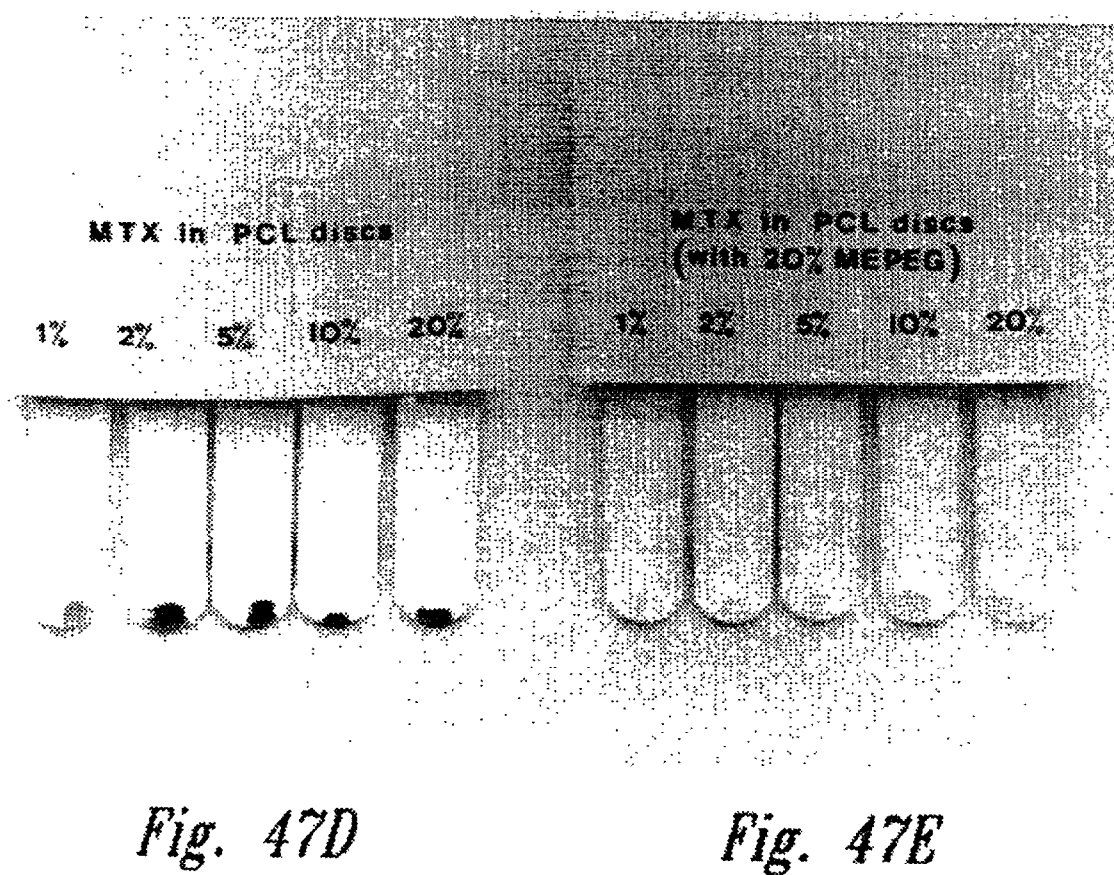
*Fig. 43A**Fig. 43B*

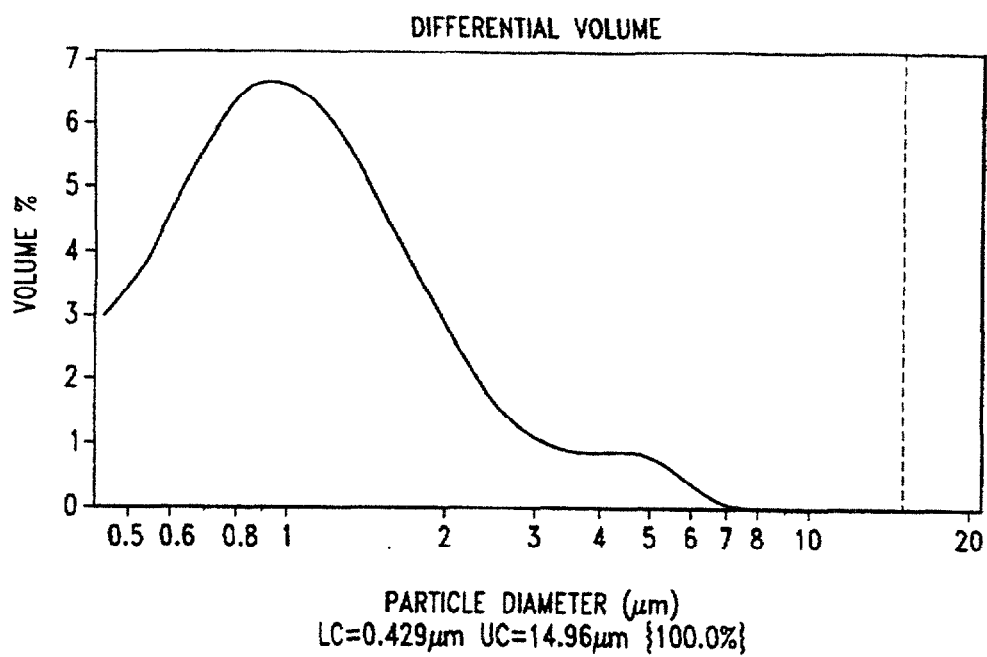
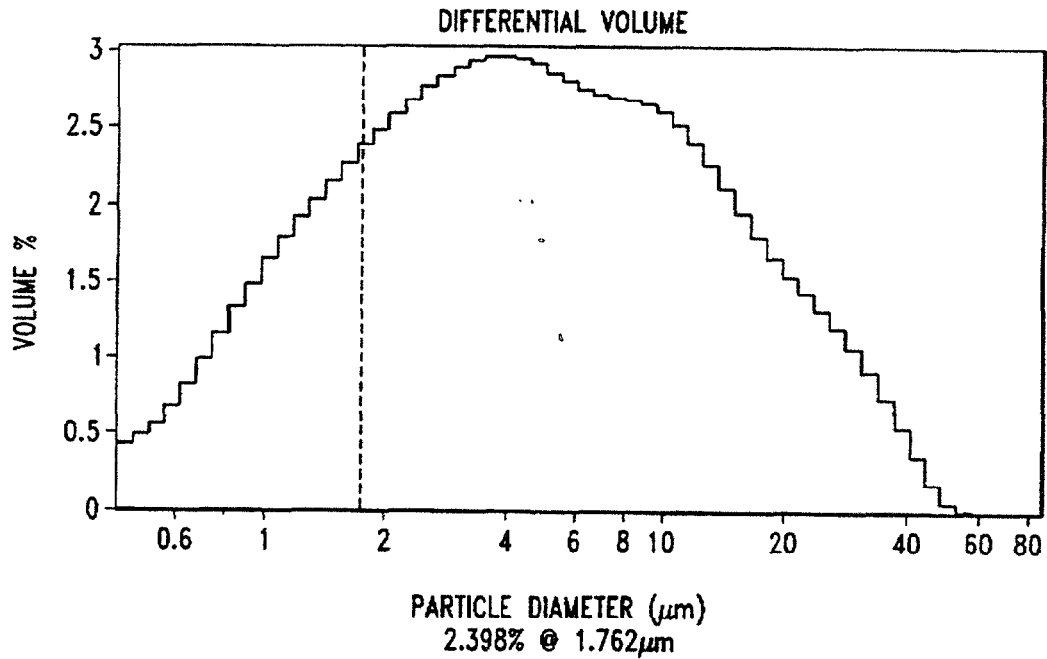
*Fig. 44A**Fig. 44B*

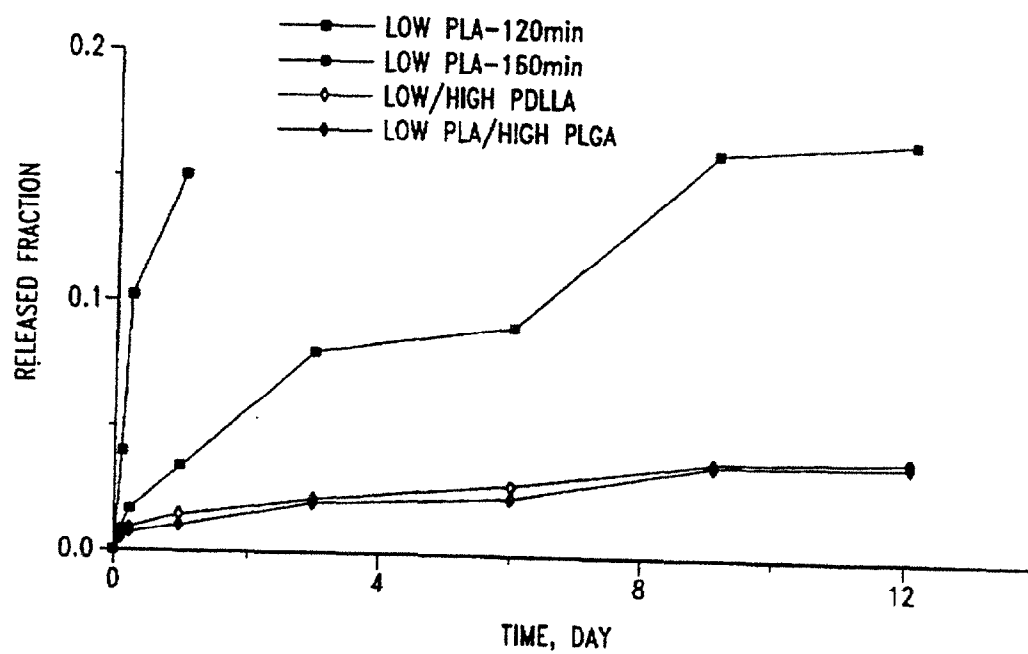
*Fig. 45**Fig. 46*

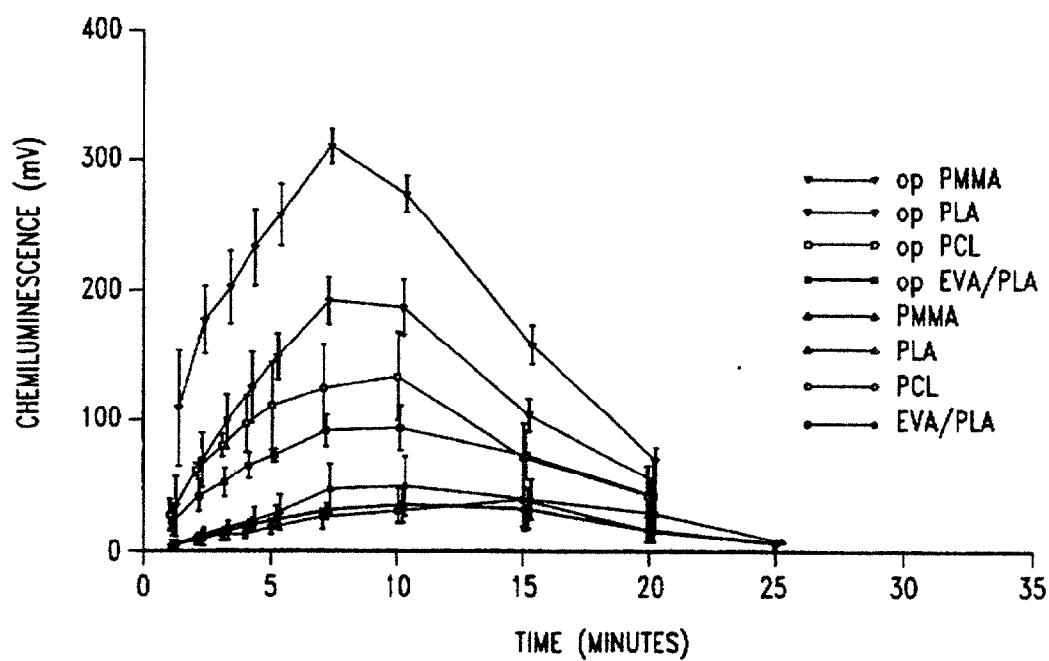
*Fig. 47A**Fig. 47B*

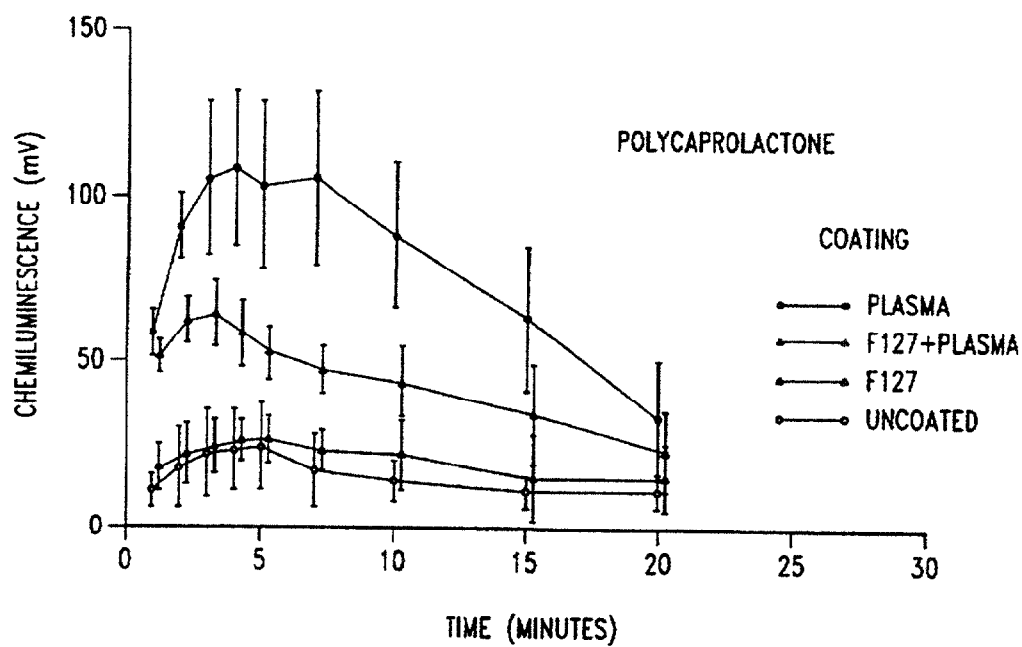
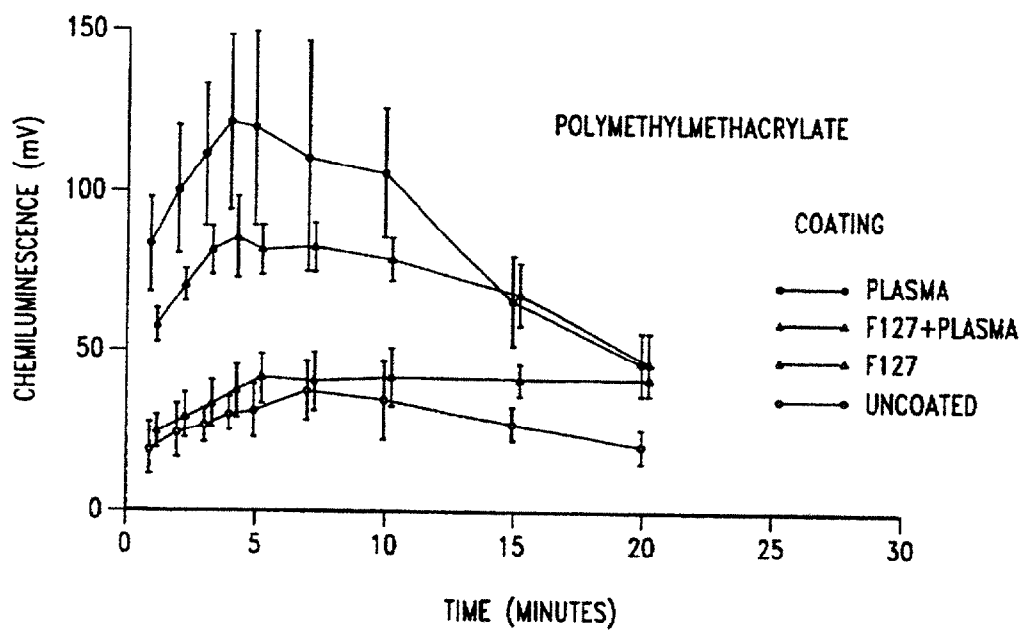
*Fig. 47C*

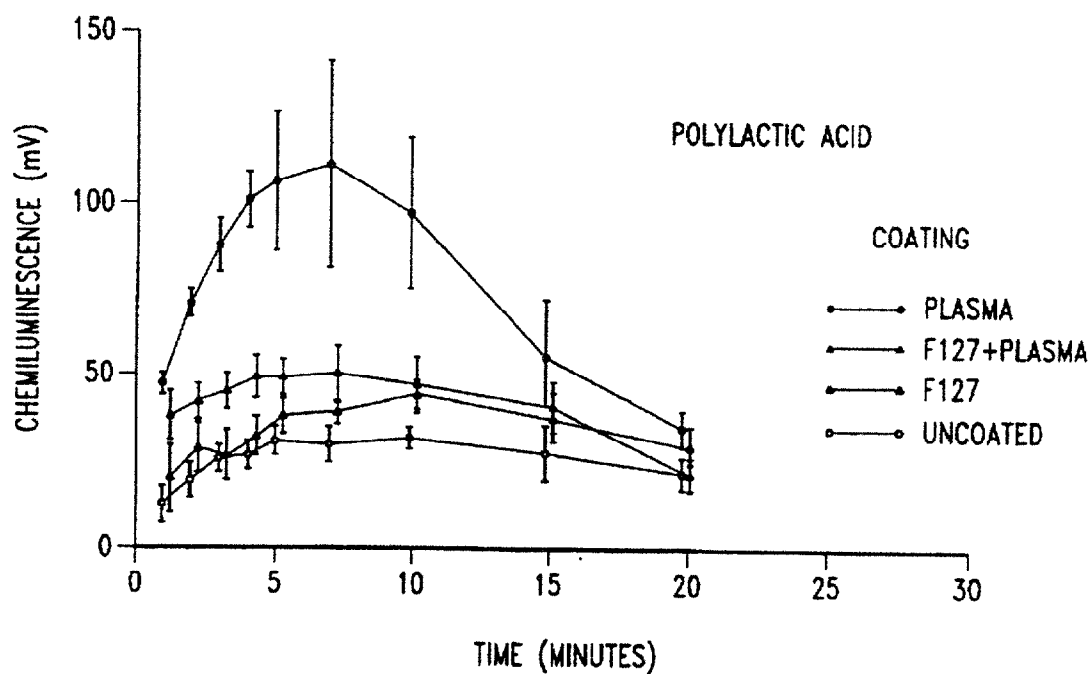
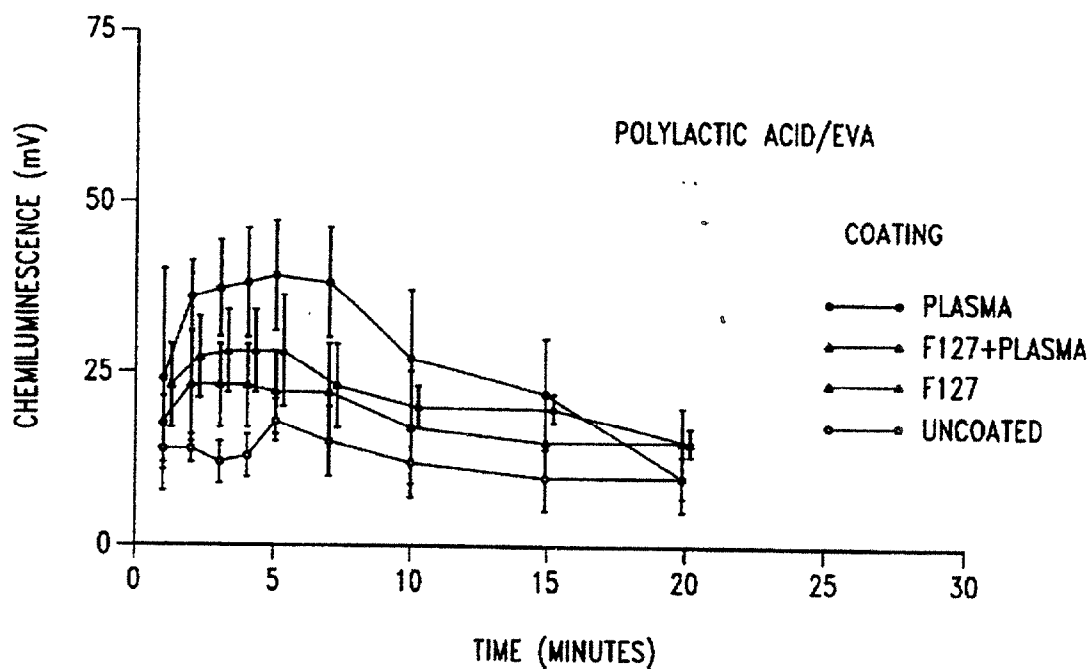


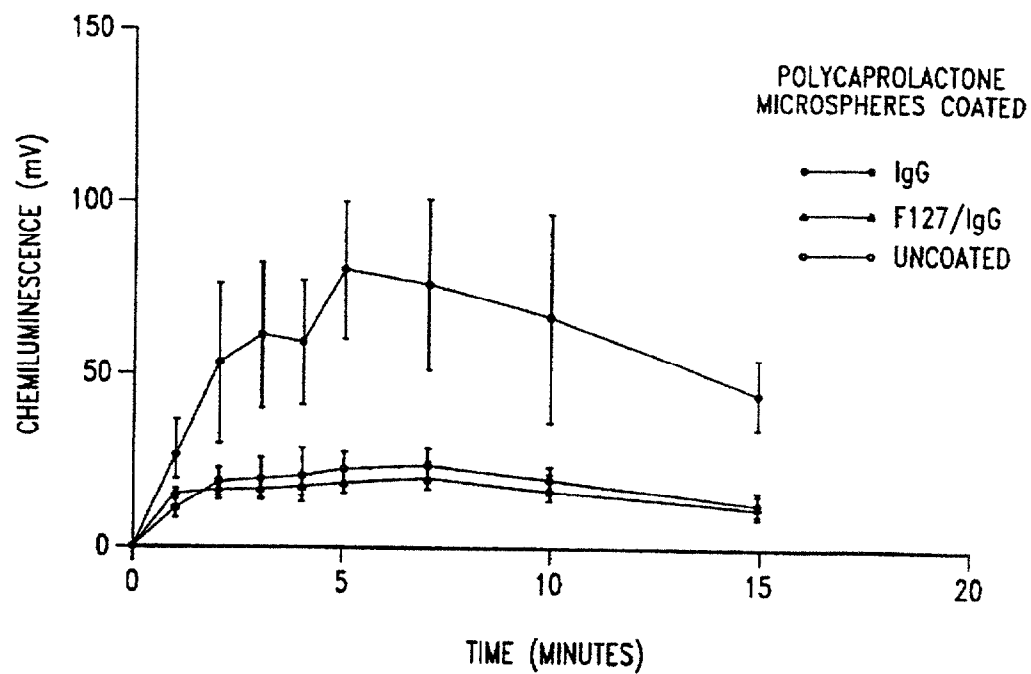
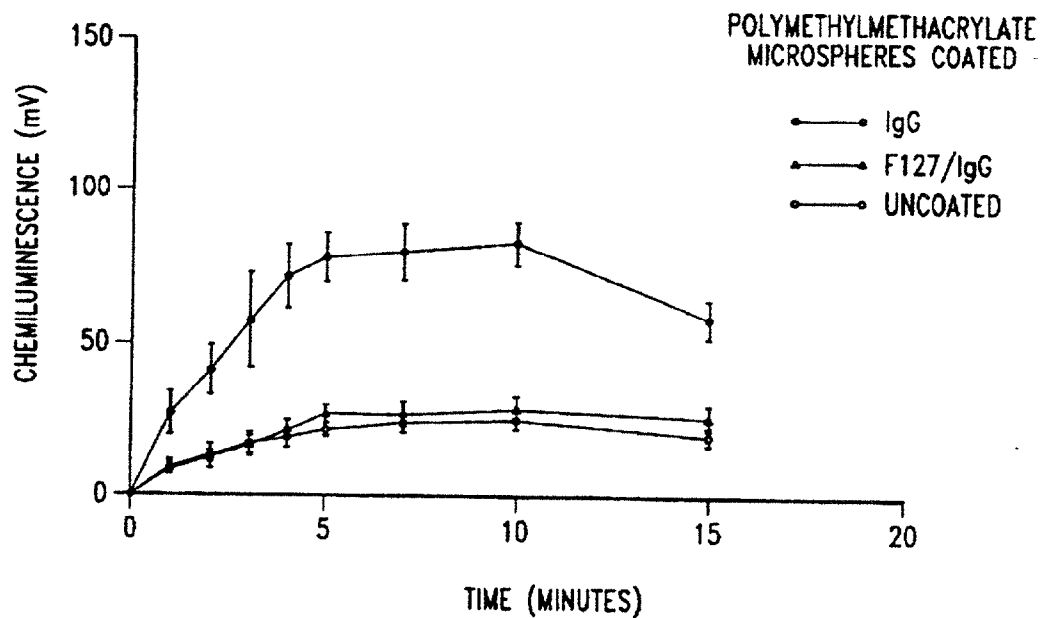
*Fig. 48**Fig. 49*

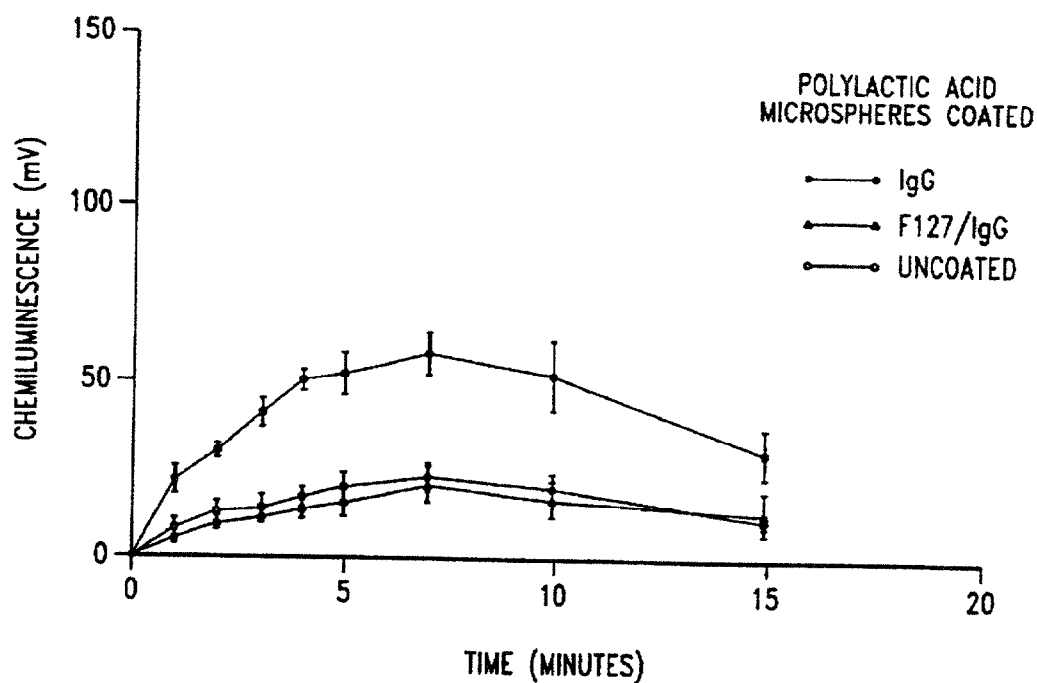
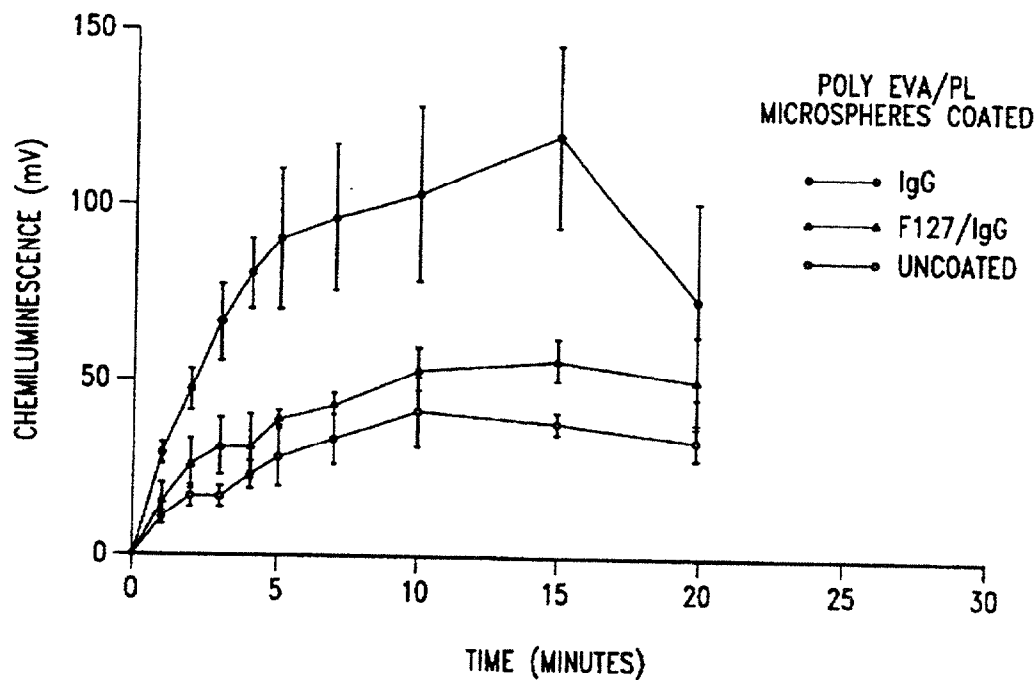
*Fig. 50*

*Fig. 51*

*Fig. 52**Fig. 53*

*Fig. 54**Fig. 55*

*Fig. 56**Fig. 57*

*Fig. 58**Fig. 59*

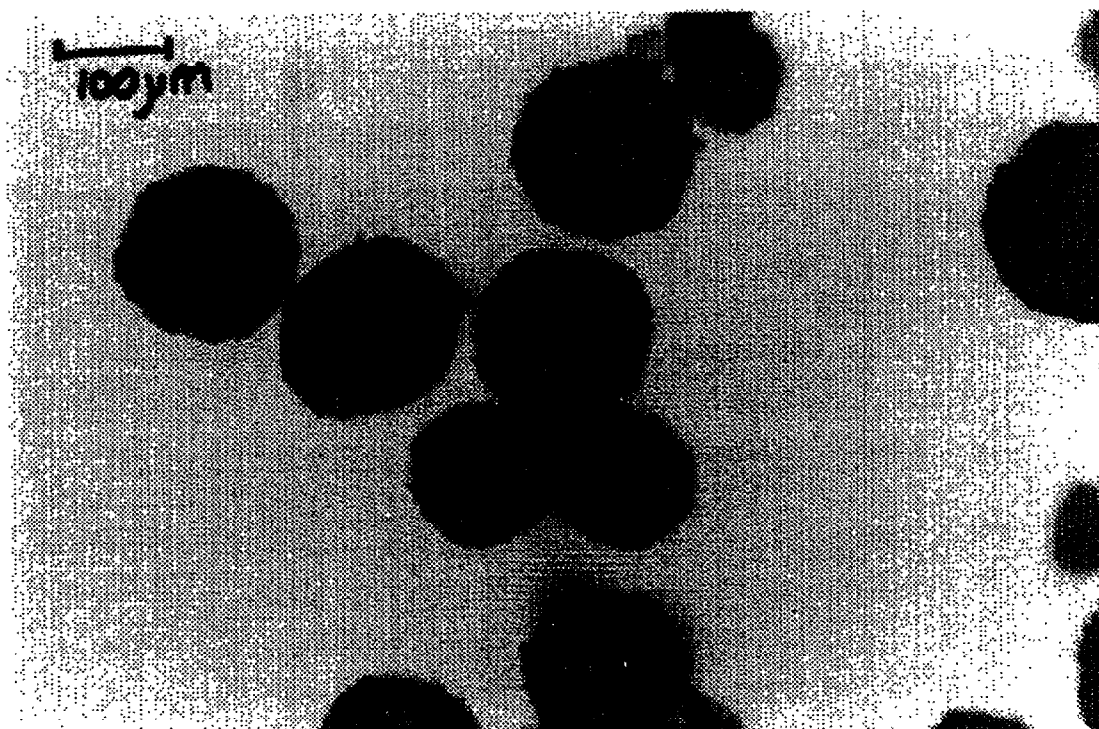
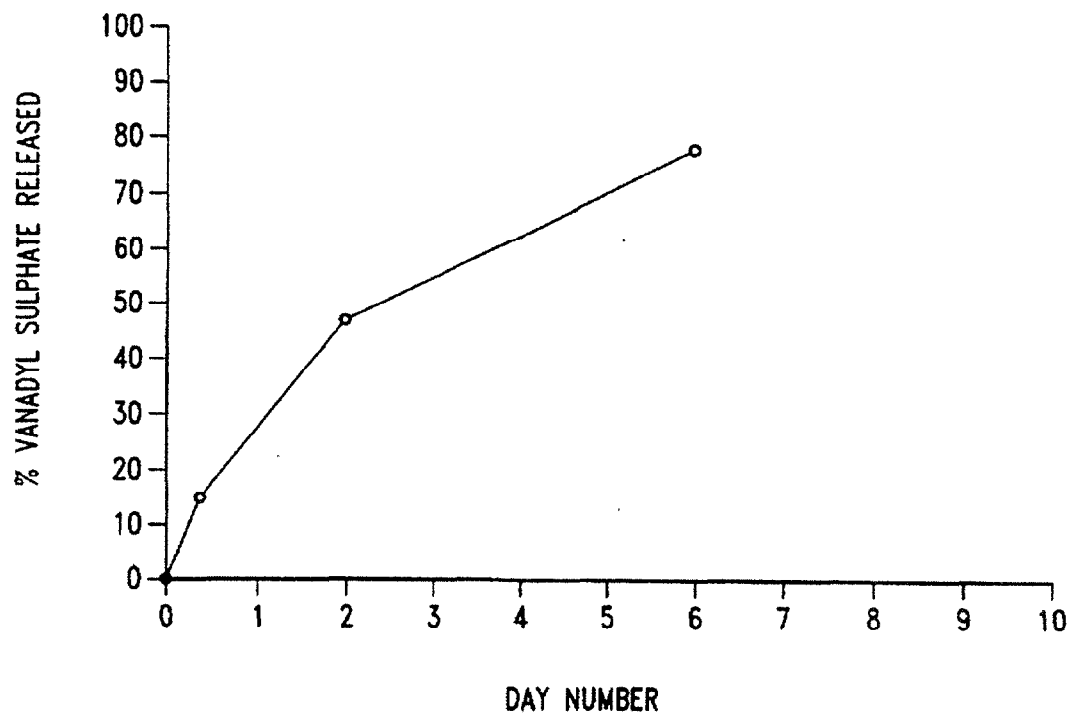


Fig. 60

*Fig. 61*

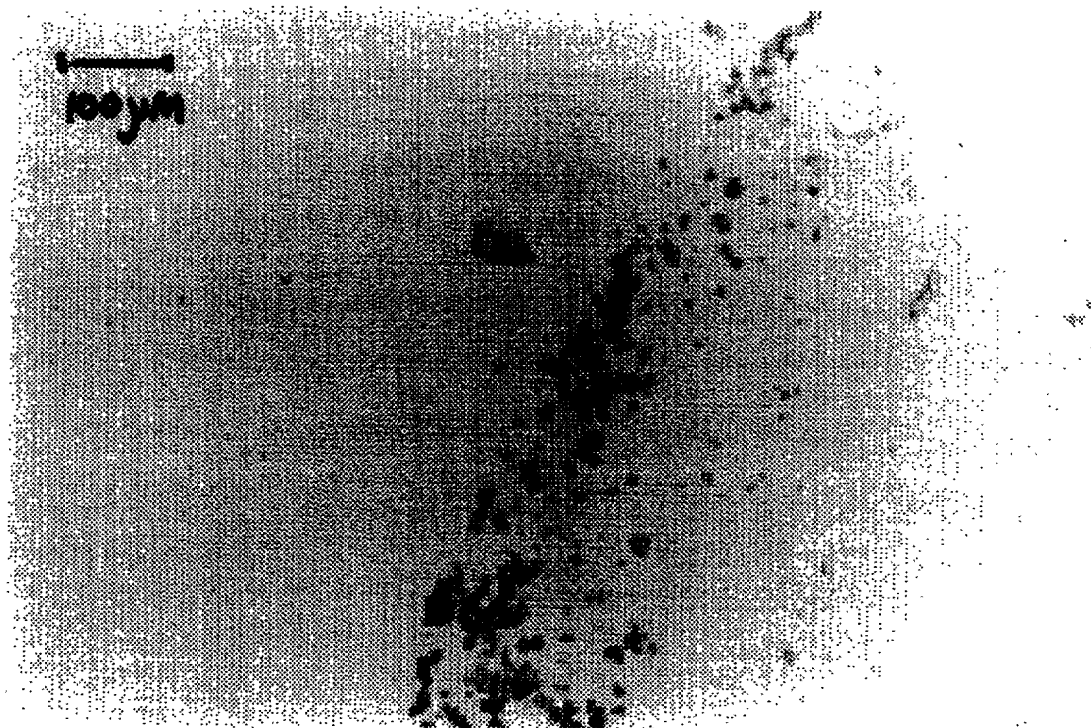
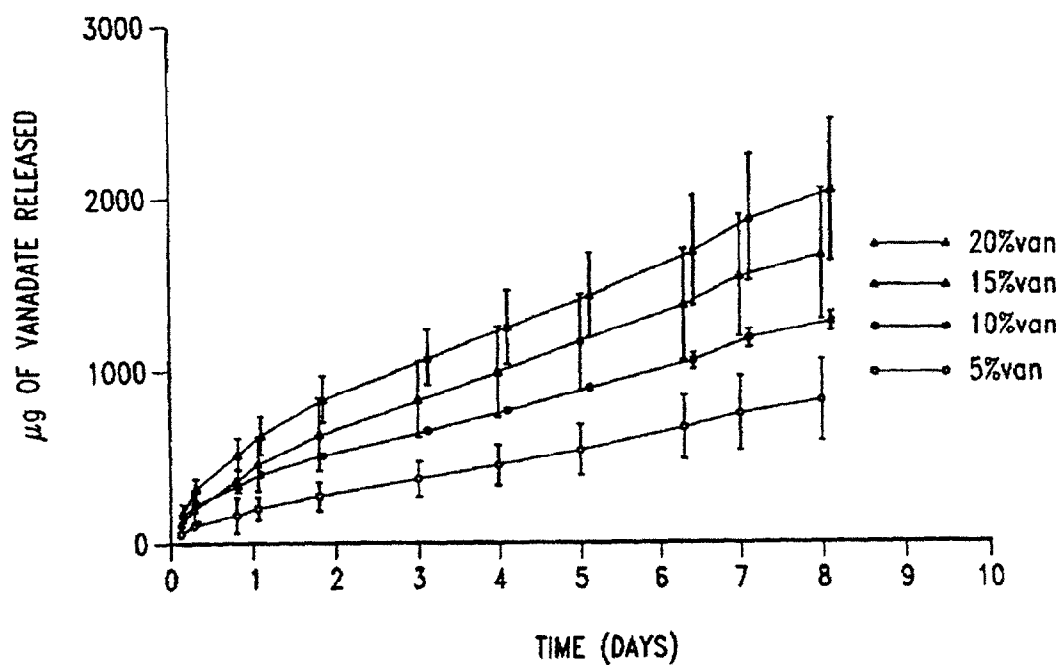
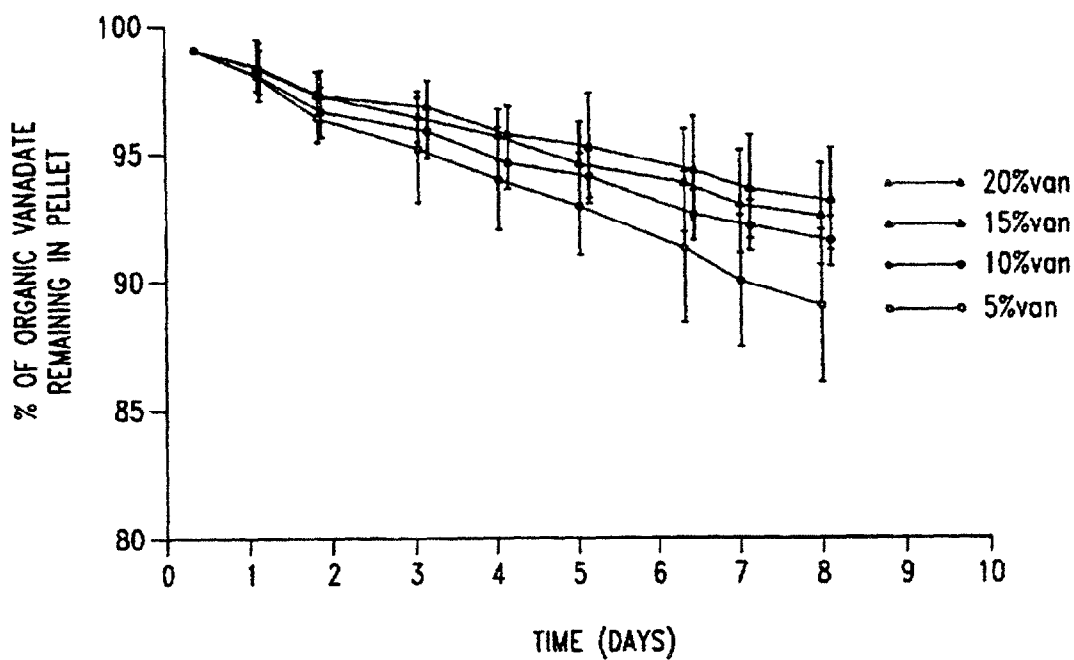


Fig. 62

*Fig. 63A**Fig. 63B*

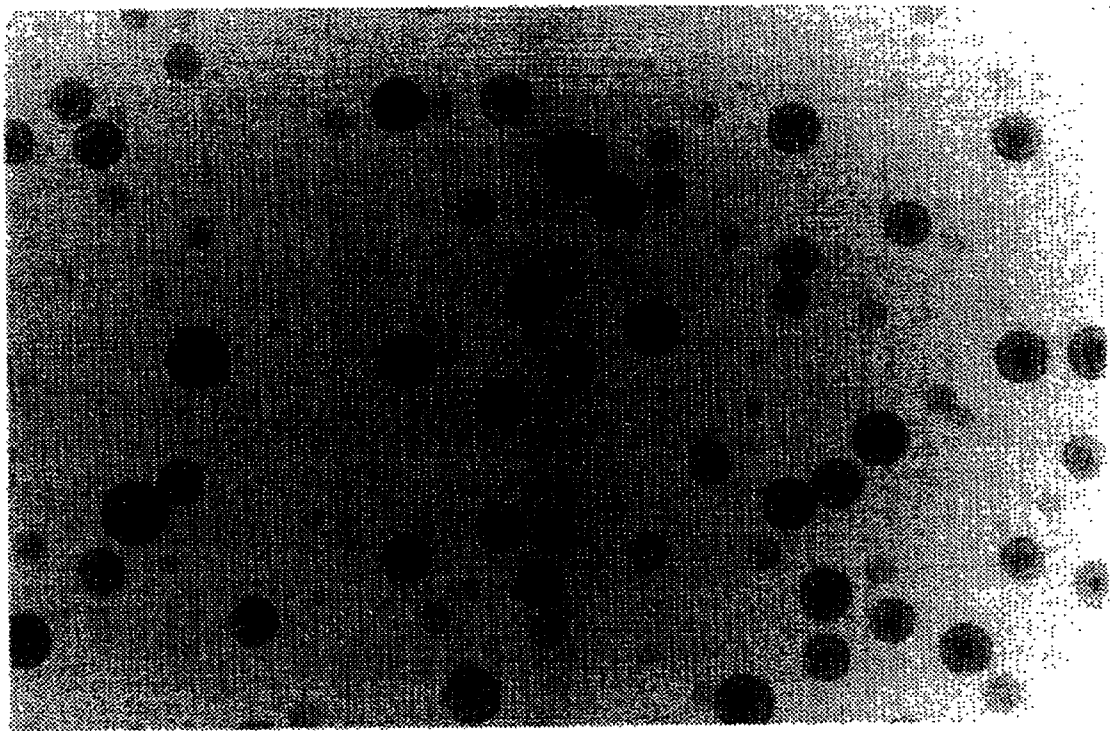


Fig. 64

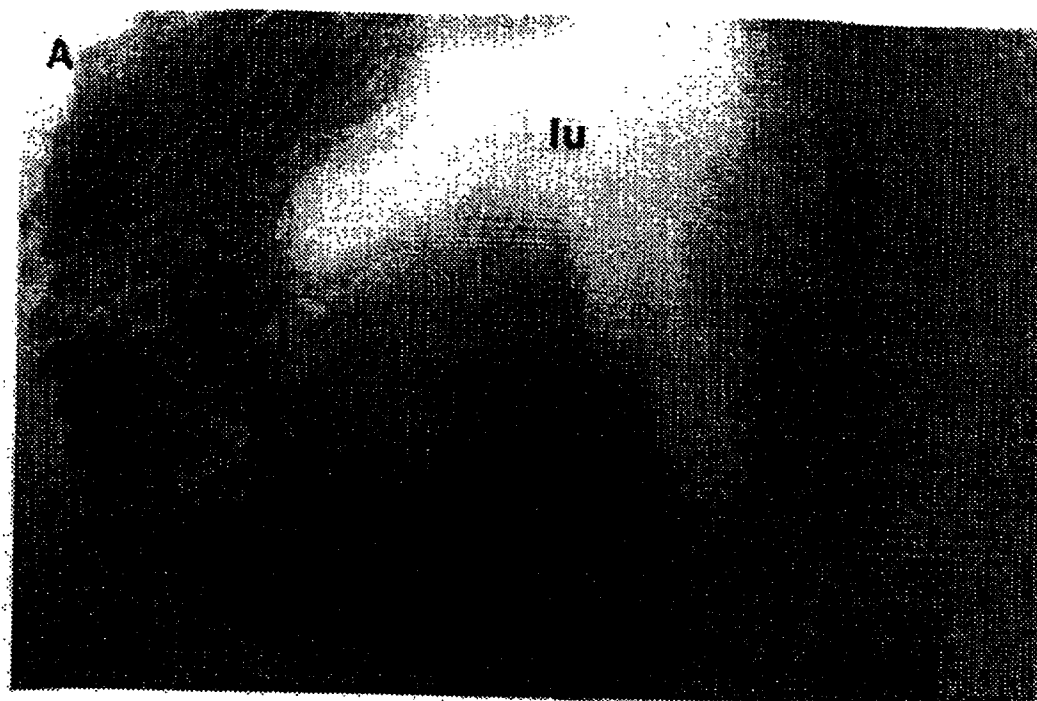


Fig. 65A



Fig. 65B

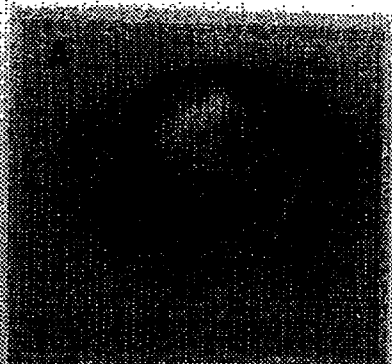


Fig. 66A

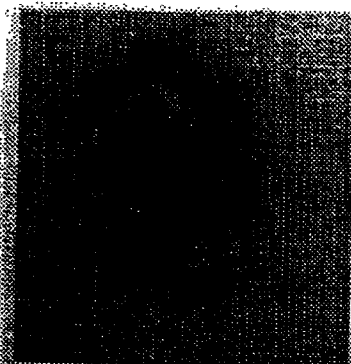


Fig. 66C



Fig. 66B

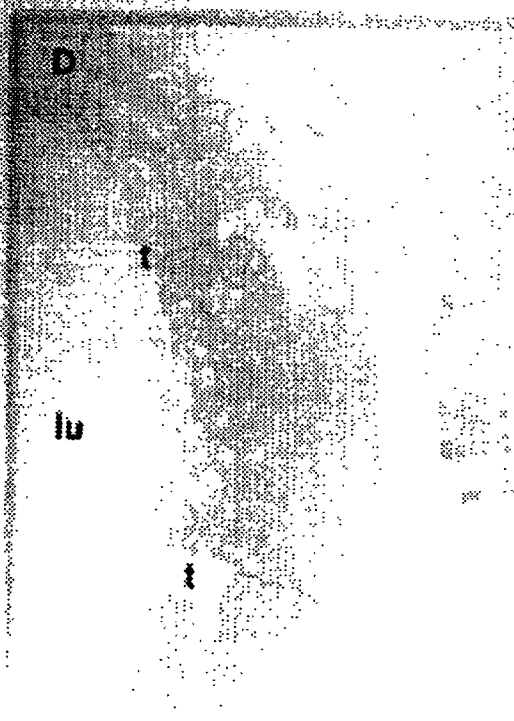


Fig. 66D

ANTI-ANGIOGENIC COMPOSITIONS AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a division of U.S. patent application Ser. No. 08/417,160, filed Apr. 3, 1995, now abandoned; which is a continuation-in-part of U.S. patent application Ser. No. 08/094,536, filed Jul. 19, 1993, now abandoned.

TECHNICAL FIELD

The present invention relates generally to compositions and methods for treating cancer and other angiogenic-dependent diseases, and more specifically, to compositions comprising anti-angiogenic factors and polymeric carriers, stents which have been coated with such compositions, as well as method for utilizing these stents and compositions.

BACKGROUND OF THE INVENTION

Angiogenesis-dependent diseases (i.e., those diseases which require or induce vascular growth) represent a significant portion of all diseases for which medical treatment is sought. For example, cancer is the second leading cause of death in the United States, and accounts for over one-fifth of the total mortality. Briefly, cancer is characterized by the uncontrolled division of a population of cells which, most typically, leads to the formation of one or more tumors. Such tumors are also characterized by the ingrowth of vasculature which provide various factors that permit continued tumor growth. Although cancer is generally more readily diagnosed than in the past, many forms, even if detected early, are still incurable.

A variety of methods are presently utilized to treat cancer, including for example, various surgical procedures. If treated with surgery alone however, many patients (particularly those with certain types of cancer, such as breast, brain, colon and hepatic cancer) will experience recurrence of the cancer. Therefore, in addition to surgery, many cancers are also treated with a combination of therapies involving cytotoxic chemotherapeutic drugs (e.g., vincristine, vinblastine, cisplatin, methotrexate, 5-FU, etc.) and/or radiation therapy. One difficulty with this approach, however, is that radiotherapeutic and chemotherapeutic agents are toxic to normal tissues, and often create life-threatening side effects. In addition, these approaches often have extremely high failure/remission rates.

In addition to surgical, chemo- and radiation therapies, others have attempted to utilize an individual's own immune system in order to eliminate cancerous cells. For example, some have suggested the use of bacterial or viral components as adjuvants in order to stimulate the immune system to destroy tumor cells. (See generally "Principles of Cancer Biotherapy," Oldham (ed.), Raven Press, New York, 1987.) Such agents have generally been useful as adjuvants and as nonspecific stimulants in animal tumor models, but have not as yet proved to be generally effective in humans.

Lymphokines have also been utilized in the treatment of cancer. Briefly, lymphokines are secreted by a variety of cells, and generally have an effect on specific cells in the generation of an immune response. Examples of lymphokines include Interleukins (IL)-1, -2, -3, and -4, as well as colony stimulating factors such as G-CSF, GM-CSF, and M-CSF. Recently, one group has utilized IL-2 to stimulate peripheral blood cells in order to expand and produce large quantities of cells which are cytotoxic to tumor cells (Rosenberg et al., *N. Engl. J. Med.* 313:1485-1492, 1985).

Others have suggested the use of antibodies in the treatment of cancer. Briefly, antibodies may be developed which recognize certain cell surface antigens that are either unique, or more prevalent on cancer cells compared to normal cells.

These antibodies, or "magic bullets," may be utilized either alone or conjugated with a toxin in order to specifically target and kill tumor cells (Dillman, "Antibody Therapy," *Principles of Cancer Biotherapy*, Oldham (ed.), Raven Press, Ltd., New York, 1987). However, one difficulty is that most monoclonal antibodies are of murine origin, and thus hypersensitivity against the murine antibody may limit its efficacy, particularly after repeated therapies. Common side effects include fever, sweats and chills, skin rashes, arthritis, and nerve palsies.

One additional difficulty of present methods is that local recurrence and local disease control remains a major challenge in the treatment of malignancy. In particular, a total of 630,000 patients annually (in the U.S.) have localized disease (no evidence of distant metastatic spread) at the time of presentation; this represents 64% of all those patients diagnosed with malignancy (this does not include nonmelanoma skin cancer or carcinoma in situ). For the vast majority of these patients, surgical resection of the disease represents the greatest chance for a cure and indeed 428,000 will be cured after the initial treatment—428,000. Unfortunately, 202,000 (or 32% of all patients with localized disease) will relapse after the initial treatment. Of those who relapse, the number who will relapse due to local recurrence of the disease amounts to 133,000 patients annually (or 21% of all those with localized disease). The number who will relapse due to distant metastases of the disease is 68,000 patients annually (11% of all those with localized disease). Another 102,139 patients annually will die as a direct result of an inability to control the local growth of the disease.

Nowhere is this problem more evident than in breast cancer, which affects 186,000 women annually in the U.S. and whose mortality rate has remained unchanged for 50 years. Surgical resection of the disease through radical mastectomy, modified radical mastectomy, or lumpectomy remains the mainstay of treatment for this condition. Unfortunately, 39% of those treated with lumpectomy alone will develop a recurrence of the disease, and surprisingly, so will 25% of those in which the resection margin is found to be clear of tumor histologically. As many as 90% of these local recurrences will occur within 2 cm of the previous excision site.

Similarly, in 1991, over 113,000 deaths and 238,600 new cases of liver metastasis were reported in North America alone. The mean survival time for patients with liver metastases is only 6.6 months once liver lesions have developed. Non-surgical treatment for hepatic metastases include systemic chemotherapy, radiation, chemoembolization, hepatic arterial chemotherapy, and intraarterial radiation. However, despite evidence that such treatments can transiently decrease the size of the hepatic lesions (e.g., systemic chemotherapy and hepatic arterial chemotherapy initially reduces lesions in 15-20%, and 80% of patients, respectively), the lesions invariably reoccur. Surgical resection of liver metastases represents the only possibility for a cure, but such a procedure is possible in only 5% of patients with metastases, and in only 15-20% of patients with primary hepatic cancer.

One method that has been attempted for the treatment of tumors with limited success is therapeutic embolization. Briefly, blood vessels which nourish a tumor are deliberately blocked by injection of an embolic material into the vessels. A variety of materials have been attempted in this regard.

including autologous substances such as fat, blood clot, and chopped muscle fragments, as well as artificial materials such as wool, cotton, steel balls, plastic or glass beads, tantalum powder, silicone compounds, radioactive particles, sterile absorbable gelatin sponge (Sterispon, Gelfoam), oxidized cellulose (Oxycel), steel coils, alcohol, lyophilized human dura mater (Lyodura), microfibrillar collagen (Avitene), collagen fibrils (Tachotop), polyvinyl alcohol sponge (PVA; Ivalon), Barium-impregnated silicon spheres (Biss) and detachable balloons. The size of liver metastases may be temporarily decreased utilizing such methods, but tumors typically respond by causing the growth of new blood vessels into the tumor.

A related problem to tumor formation is the development of cancerous blockages which inhibit the flow of material through body passageways, such as the bile ducts, trachea, esophagus, vasculature and urethra. One device, the stent, has been developed in order to hold open passageways which have been blocked by tumors or other substances. Representative examples of common stents include the Wallstent, Strecker stent, Gianturco stent, and the Palmaz stent. The major problem with stents, however, is that they do not prevent the ingrowth of tumor or inflammatory material through the interstices of the stent. If this material reaches the inside of a stent and compromises the stent lumen, it may result in blockage of the body passageway into which it has been inserted. In addition, presence of a stent in the body may induce reactive or inflammatory tissue (e.g., blood vessels, fibroblasts, white blood cells) to enter the stent lumen, resulting in partial or complete closure of the stent.

The present invention provides compositions and methods suitable for treating cancers, as well as other non-tumorigenic angiogenesis-dependent diseases, and further provides other related advantages.

SUMMARY OF THE INVENTION

Briefly stated, the present invention provides anti-angiogenic compositions, as well as methods and devices which utilize such compositions for the treatment of cancer and other angiogenesis-dependent diseases. Within one aspect of the present invention, compositions are provided (anti-angiogenic compositions) comprising (a) an anti-angiogenic factor and (b) a polymeric carrier. A wide variety of molecules may be utilized within the scope of the present invention as anti-angiogenic factors, including for example Anti-Invasive Factor, retinoic acids and their derivatives, paclitaxel including analogues and derivatives thereof, Suramin, Tissue Inhibitor of Metalloproteinase-1, Tissue Inhibitor of Metalloproteinase-2, Plasminogen Activator Inhibitor-1 and Plasminogen Activator Inhibitor-2, and lighter "d group" transition metals. Similarly, a wide variety of polymeric carriers may be utilized, representative examples of which include poly (ethylene-vinyl acetate) (40% cross-linked), poly (D,L-lactic acid) oligomers and polymers, poly (L-lactic acid) oligomers and polymers, poly (glycolic acid), copolymers of lactic acid and glycolic acid, poly (caprolactone), poly (valerolactone), poly (anhydrides), copolymers of poly (caprolactone) or poly (lactic acid) with polyethylene glycol, and blends thereof.

Within certain preferred embodiments, the compositions comprise a compound which disrupts microtubule function, such as, for example, paclitaxel, estramustine, colchicine, methotrexate, curacin-A, epothilone, vinblastine or tBCEV. Within other preferred embodiments, the compositions comprise a polymeric carrier and a lighter d group transition

metal (e.g., a vanadium species, molybdenum species, tungsten species, titanium species, niobium species or tantalum species) which inhibits the formation of new blood vessels.

Within one embodiment of the invention, the composition has an average size of 15 to 200 μm . Within other embodiments, the polymeric carrier of the composition has a molecular weight ranging from less than 1,000 daltons to greater than 200,000 to 300,000 daltons. Within yet other embodiments, the compositions provided herein may be formed into films with a thickness of between 100 μm and 2 mm, or thermologically active compositions which are liquid at one temperature (e.g., above 45° C.) and solid or semi-solid at another (e.g., 37° C.).

Within another aspect of the present invention methods for embolizing a blood vessel are provided, comprising the step of delivering into the vessel a therapeutically effective amount of an anti-angiogenic composition (as described above), such that the blood vessel is effectively occluded. Within one embodiment, the anti-angiogenic composition is delivered to a blood vessel which nourishes a tumor.

Within yet another aspect of the present invention, stents are provided comprising a generally tubular structure, the surface being coated with one or more anti-angiogenic compositions. Within other aspects of the present invention, methods are provided for expanding the lumen of a body passageway, comprising inserting a stent into the passageway, the stent having a generally tubular structure, the surface of the structure being coated with an anti-angiogenic composition as described above, such that the passageway is expanded. Within various embodiments of the invention, methods are provided for eliminating biliary obstructions, comprising inserting a biliary stent into a biliary passageway; for eliminating urethral obstructions, comprising inserting a urethral stent into a urethra; for eliminating esophageal obstructions, comprising inserting an esophageal stent into an esophagus; and for eliminating tracheal/bronchial obstructions, comprising inserting a tracheal/bronchial stent into the trachea or bronchi. In each of these embodiments, the stent has a generally tubular structure, the surface of which is coated with an anti-angiogenic composition as described above.

Within another aspect of the present invention, methods are provided for treating tumor excision sites, comprising administering an anti-angiogenic composition as described above to the resection margins of a tumor subsequent to excision, such that the local recurrence of cancer and the formation of new blood vessels at the site is inhibited. Within yet another aspect of the invention, methods for treating corneal neovascularization are provided, comprising the step of administering to a patient a therapeutically effective amount of an anti-angiogenic composition as described above to the cornea, such that the formation of blood vessels is inhibited. Within one embodiment, the anti-angiogenic composition further comprises a topical corticosteroid.

Within another aspect of the present invention, methods are provided for inhibiting angiogenesis in patients with non-tumorigenic, angiogenesis-dependent diseases, comprising administering to a patient a therapeutically effective amount of paclitaxel to a patient with a non-tumorigenic angiogenesis-dependent disease, such that the formation of new blood vessels is inhibited. Within other aspects, methods are provided for embolizing blood vessels in non-tumorigenic, angiogenesis-dependent diseases, comprising delivering to the vessel a therapeutically effective amount of a composition comprising paclitaxel, such that the blood vessel is effectively occluded.

Within yet other aspects of the present invention, methods are provided for expanding the lumen of a body passageway, comprising inserting a stent into the passageway, the stent having a generally tubular structure, the surface of the structure being coated with a composition comprising paclitaxel, such that the passageway is expanded. Within various embodiments of the invention, methods are provided for eliminating biliary obstructions, comprising inserting a biliary stent into a biliary passageway; for eliminating urethral obstructions, comprising inserting a urethral stent into a urethra; for eliminating esophageal obstructions, comprising inserting an esophageal stent into an esophagus; and for eliminating tracheal/bronchial obstructions, comprising inserting a tracheal/bronchial stent into the trachea or bronchi. Within each of these embodiments the stent has a generally tubular structure, the surface of the structure being coated with a composition comprising paclitaxel.

Within another aspect of the present invention, methods are provided for treating a tumor excision site, comprising administering a composition comprising paclitaxel to the resection margin of a tumor subsequent to excision, such that the local recurrence of cancer and the formation of new blood vessels at the site is inhibited. Within other aspects, methods are provided for treating neovascular diseases of the eye, comprising administering to a patient a therapeutically effective amount of an anti-angiogenic factor (such as a compound which disrupts microtubule function) to the eye, such that the formation of new vessels is inhibited.

Within other aspects of the present invention, methods are provided for treating inflammatory arthritis, comprising administering to a patient a therapeutically effective amount of an anti-angiogenic factor (such as a compound which disrupts microtubule function), or a composition comprising an anti-angiogenic factor and a polymeric carrier to a joint. Within preferred embodiments, the anti-angiogenic factor may be a compound which disrupts microtubule function such as paclitaxel, or an element from the lighter 'd group' transition metals, such as a vanadium species.

Within yet another aspect of the invention, pharmaceutical products are provided, comprising (a) a compound which disrupts microtubule function, in a container, and (b) a notice associated with the container in form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals, which notice is reflective of approval by the agency of a compound which disrupts microtubule function, for human or veterinary administration to treat non-tumorigenic angiogenesis-dependent diseases such as, for example, inflammatory arthritis or neovascular diseases of the eye. Briefly, Federal Law requires that the use of a pharmaceutical agent in the therapy of humans be approved by an agency of the Federal government. Responsibility for enforcement (in the United States) is with the Food and Drug Administration, which issues appropriate regulations for securing such approval, detailed in 21 U.S.C. §§301-392. Regulation for biological materials comprising products made from the tissues of animals, is also provided under 42 U.S.C. §262. Similar approval is required by most countries, although, regulations may vary from country to country.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures, devices or compositions, and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a photograph which shows a shell-less egg culture on day 6. FIG. 1B is a digitized computer-displayed

image taken with a stereomicroscope of living, unstained capillaries (1040×). FIG. 1C is a photograph of a corrosion casting which shows CAM microvasculature that are fed by larger, underlying vessels. (arrows; 1300×). FIG. 1D is a photograph which depicts a 0.5 mm thick plastic section cut transversely through the CAM, and recorded at the light microscope level. This photograph shows the composition of the CAM, including an outer double-layered ectoderm (Ec), a mesoderm (M) containing capillaries (arrows) and scattered adventitia cells, and a single layered endoderm (En) (400×). FIG. 1E is a photograph at the electron microscope level (3500×) wherein typical capillary structure is presented showing thin-walled endothelial cells (arrowheads) and an associated pericyte.

FIGS. 2A, 2B, 2C and 2D are a series of digitized images of four different, unstained CAMs taken after a 48 hour exposure to digitized images of four different living, unstained CAMs were taken after a 48 h exposure to 10μ paclitaxel per 10 ml of methylcellulose. The transparent methylcellulose disk (*) containing paclitaxel is present on each CAM and is positioned over a singular avascular zone (A) with surrounding blood islands (Is). These avascular areas extend beyond the disk and typically have a diameter of 6 mm. FIG. 2D illustrates the typical "elbowing" effect (arrowheads) of both small and large vessels being redirected away from the periphery of the avascular zone.

FIG. 3A is a photograph (Mag=400×) which shows just peripheral to the avascular zone, that capillaries (arrowheads) exhibit numerous endothelial cells arrested in mitosis. Ectoderm (Ec); Mesoderm (M); Endoderm (En). FIG. 3B (Mag=400×) shows that within the avascular zone proper the typical capillary structure has been eliminated and there are numerous extravasated blood cells (arrowheads). FIG. 3C (Mag=400×) shows that in the central area of the avascular zone, red blood cells are dispersed throughout the mesoderm.

FIGS. 3A, 3B and 3C are a series of photographs of 0.5 mm thick plastic sections transversely cut through a paclitaxel-treated CAM at three different locations within the avascular zone.

FIGS. 4A, 4B and 4C are series of electron micrographs which were taken from locations similar to that of FIGS. 3A, 3B and 3C (respectively) above.

FIG. 4A (Mag=2,200×) shows a small capillary lying subjacent to the ectodermal layer (Ec) possessing three endothelial cells arrested in mitosis (*). Several other cell types in both the ectoderm and mesoderm are also arrested in mitosis. FIG. 4B (Mag=2,800×) shows the early avascular phase contains extravasated blood cells subjacent to the ectoderm; these blood cells are intermixed with presumptive endothelial cells (*) and their processes. Degradative cellular vacuoles (arrowhead). FIG. 4C (Mag=2,800×) shows that in response to paclitaxel, the ecto-mesodermal interface has become populated with cells in various stages of degradation containing dense vacuoles and granules (arrowheads).

FIG. 5 is a bar graph which depicts the size distribution of microspheres by number (5% poly (ethylene-vinyl acetate) with 10 mg sodium suramin into 5% PVA).

FIG. 6 is a bar graph which depicts the size distribution of microspheres by weight (5% poly (ethylene-vinyl acetate) with 10 mg sodium suramin into 5% PVA).

FIG. 7 is a graph which depicts the weight of encapsulation of Sodium Suramin in 50 mg poly (ethylene-vinyl acetate).

FIG. 8 is a graph which depicts the percent of encapsulation of Sodium Suramin in 50 mg poly (ethylene-vinyl acetate).

FIG. 9 is a bar graph which depicts the size distribution by weight of 5% ELVAX microspheres containing 10 mg sodium suramin made in 5% PVA containing 10% NaCl.

FIG. 10 is a bar graph which depicts the size distribution by weight of 5% microspheres containing 10 mg sodium suramin made in 5% PVA containing 10% NaCl.

FIG. 11 is a bar graph which depicts the size distribution by number of 5% microspheres containing 10 mg sodium suramin made in 5% PVA containing 10% NaCl.

FIG. 12 is a line graph which depicts the time course of sodium suramin release.

FIG. 13 is an illustration of a representative embodiment of hepatic tumor embolization.

FIG. 14 is an illustration of the insertion of a representative stent coated with an anti-angiogenic composition.

FIG. 15A is a graph which shows the effect of the EVA:PLA polymer blend ratio upon aggregation of microspheres. FIG. 15B is a scanning electron micrograph which shows the size of "small" microspheres. FIG. 15C (which includes a magnified inset—labelled "15C-inset") is a scanning electron micrograph which shows the size of "large" microspheres. FIG. 15D is a graph which depicts the time course of in vitro paclitaxel release from 0.6% w/v paclitaxel-loaded 50:50 EVA:PLA polymer blend microspheres into phosphate buffered saline (pH 7.4) at 37° C. Open circles are "small" sized microspheres, and closed circles are "large" sized microspheres. FIG. 15F is a photograph of a CAM which shows the results of paclitaxel release by microspheres ("MS"). FIG. 15F is a photograph similar to that of 15E at increased magnification.

FIG. 16A is a graph which shows release rate profiles from polycaprolactone microspheres containing 1%, 2%, 5% or 10% paclitaxel into phosphate buffered saline at 37° C. FIG. 16B is a photograph which shows a CAM treated with control microspheres. FIG. 16C is a photograph which shows a CAM treated with 5% paclitaxel loaded microspheres.

FIGS. 17A and 17B, respectively, are two graphs which show the release of paclitaxel from EVA films, and the percent paclitaxel remaining in those same films over time. FIG. 17C is a graph which shows the swelling of EVA/F127 films with no paclitaxel over time. FIG. 17D is a graph which shows the swelling of EVA/Span 80 films with no paclitaxel over time. FIG. 17E is a graph which depicts a stress vs. strain curve for various EVA/F127 blends.

FIGS. 18A and 18B are two graphs which show the melting point of PCL/MePEG polymer blends as a function of % MePEG in the formulation (18A), and the percent increase in time needed for PCL paste at 60° C. to being to solidify as a function of the amount of MePEG in the formulation (18B). FIG. 18C is a graph which depicts the softness of varying PCL/MePEG polymer blends. FIG. 18D is a graph which shows the percent weight change over time for polymer blends of various MePEG concentrations. FIG. 18E is a graph which depicts the rate of paclitaxel release over time from various polymer blends loaded with 1% paclitaxel. FIGS. 18F and 18G are graphs which depict the effect of varying quantities of paclitaxel on the total amount of paclitaxel released from a 20% MePEG/PCL blend. FIG. 18H is a graph which depicts the effect of MePEG on the tensile strength of a MePEG/PCL polymer.

FIG. 19A is a photograph which shows control (unloaded) thermopaste on a CAM. Note that both large vessels and small vessels (capillaries) are found immediately adjacent to the paste. Blood flow in the area around and under the paste

is unaffected. FIG. 19B is a photograph of 20% paclitaxel-loaded thermopaste on a CAM. Note the disruption of the vasculature when compared to the surrounding tissues. The drug loaded paste has blocked the growth of the capillaries, caused regression of the larger vessels, and created a region of avascularity on the CAM assay. FIG. 19C is a photograph of 0.5% paclitaxel-loaded thermopaste on a CAM (Mag.—40×). Briefly, the paclitaxel-loaded thermopaste disk induced an avascular zone measuring 6 mm in diameter on the CAM. This avascular region was induced by blocking new capillary growth and occluding, disrupting, and regressing the existing blood vessels found within the treated region. FIG. 19D is a photograph of control (unloaded) Thermopaste on a CAM. Briefly, after a 2 day exposure, the blood vessel organization of the CAM (Mag=50×) treated with the control paste shows normal blood vessel organization. Functional vessels are located immediately adjacent to the unloaded paste.

FIGS. 20A and 20B are two photographs of a CAM having a tumor treated with control (unloaded) thermopaste. Briefly, in FIG. 20 A the central white mass is the tumor tissue. Note the abundance of blood vessels entering the tumor from the CAM in all directions. The tumor induces the ingrowth of the host vasculature through the production of "angiogenic factors." The tumor tissue expands distally along the blood vessels which supply it. FIG. 20B is an underside view of the CAM shown in 20A. Briefly, this view demonstrates the radial appearance of the blood vessels which enter the tumor like the spokes of a wheel. Note that the blood vessel density is greater in the vicinity of the tumor than it is in the surrounding normal CAM tissue. FIGS. 20C and 20D are two photographs of a CAM having a tumor treated with 20% paclitaxel-loaded thermopaste. Briefly, in FIG. 20C the central white mass is the tumor tissue. Note the paucity of blood vessels in the vicinity of the tumor tissue. The sustained release of the angiogenesis inhibitor is capable of overcoming the angiogenic stimulus produced by the tumor. The tumor itself is poorly vascularized and is progressively decreasing in size. FIG. 20D is taken from the underside of the CAM shown in 20C, and demonstrates the disruption of blood flow into the tumor when compared to control tumor tissue. Note that the blood vessel density is reduced in the vicinity of the tumor and is sparser than that of the normal surrounding CAM tissue.

FIG. 21A is a graph which shows the effect of paclitaxel/PCL on tumor growth. FIGS. 21B and 21C are two photographs which show the effect of control, 10%, and 20% paclitaxel-loaded thermopaste on tumor growth.

FIG. 22A is a photograph of synovium from a PBS injected joint. FIG. 22B is a photograph of synovium from a microsphere injected joint. FIG. 22C is a photograph of cartilage from joints injected with PBS, and FIG. 22D is a photograph of cartilage from joints injected with microspheres.

FIG. 23A is a photograph of a 0.3% Paclitaxel Ophthalmic Drop Suspension on a CAM (Mag=32×). The plastic ring was used to localize the drug treatment to the CAM. Note the lack of blood vessels located within and immediately adjacent to the ring. The functional blood vessels bordering the avascular zone are defined by their "elbowing" morphology away from the drug source. FIG. 23B is a photograph of a control (unloaded) Ophthalmic Drop Suspension on a CAM (Mag=32×). Note the normal organization of the CAM blood vessels and the abundance of functional vessels located within the ring.

FIG. 24A is a photograph of a 2.5% Paclitaxel-Loaded Stent Coating (Mag=26×). Briefly, the blood vessels sur-

rounding the avascular zone are morphologically redirected away from the paclitaxel source; this produces an avascular zone which can measure up to 6 mm in diameter. The disrupted vascular remnants which represent vascular regression can be seen within the avascular zone. FIG. 24B is a control (unloaded) Stent Coating (Mag=26 \times). Briefly, the blood vessels of the CAM are found immediately adjacent to the stent and do not illustrate any morphological alterations.

FIG. 25 is a photograph of a control stent. Briefly, this image shows the longitudinal orientation of a nylon stent incorporated within gliosarcoma tissue of the rat liver. Ingrowth within the nylon stent is evident.

FIG. 26 is a photograph of a control stent. Briefly, this image also illustrates tumor ingrowth within the lumen of the nylon stent.

FIG. 27 is a photograph of a lung. Briefly, in addition to large liver tumors, metastasis to the lung is common. Such metastases are evident by the presence of small white lobules seen throughout the lung.

FIG. 28A is a photograph of Suramin and Cortisone Acetate on a CAM (Mag=8 \times). Briefly, this image shows an avascular zone treated with 20 μ g of suramin and 70 μ g of cortisone acetate in 0.5% methylcellulose. Note the blood vessels located at the periphery of the avascular zone which are being redirected away from the drug source. FIG. 28B is a photograph which shows the vascular detail of the effected region at a higher magnification (Mag=20 \times). Note the avascular regions and the typical "elbowing" effect of the blood vessels bordering the avascular zone.

FIG. 29A is a graph which shows the chemiluminescence response of neutrophils (5×10^6 cells/ml) to plasma opsonized CPPD crystals (50 mg/ml). Effect of paclitaxel at (o) no paclitaxel, (*) 4.5 μ M, (Δ) 14 μ M, (\blacktriangle) 28 μ M, (\square) 46 μ M; n=3.

FIG. 29B is a graph which shows the time course concentration dependence of paclitaxel inhibition of plasma opsonized CPPD crystal induced neutrophil chemiluminescence.

FIG. 30A is a graph which shows superoxide anion production by neutrophils (5×10^6 cells/ml) in response to plasma opsonized CPPD crystals (50 mg/ml). Effect of paclitaxel at (o) no paclitaxel, (*) 28 μ M, (Δ) Control (cells alone); n=3. FIG. 30B is a graphic which shows the time course concentration dependence of paclitaxel inhibition of plasma opsonized CPPD crystal induced neutrophil superoxide anion production; n=3.

FIG. 31A is a graph which shows the chemiluminescence response of neutrophils (5×10^6 cells/ml) in response to plasma opsonized zymosan (1 mg/ml). Effect of paclitaxel at (o) no drug, (*) 28 μ M; n=3. FIG. 31B is a graph which shows plasma opsonized zymosan induced neutrophil superoxide anion production. Effect of paclitaxel at (o) no paclitaxel, (*) 28 μ M, (Δ) Control (cells alone); n=3.

FIG. 32A is a graph which shows myeloperoxidase release from neutrophils (5×10^6 cells/ml) in response to plasma opsonized CPPD crystals (50 mg/ml). Effect of paclitaxel at (o) no paclitaxel, (*) 28 μ M, (Δ) Control (cells alone), (\blacktriangle) Control (cells with paclitaxel at 28 μ M); n=3.

FIG. 32B is a graph which shows the concentration dependence of paclitaxel inhibition of myeloperoxidase release from neutrophils in response to plasma opsonized CPPD crystals; n=3.

FIG. 33 is a graph which shows lysozyme release from neutrophils (5×10^6 /ml) in response to plasma opsonized

CPPD crystals (50 mg/ml). Effect of paclitaxel at (o) no paclitaxel, (*) 28 μ M, (Δ) Control (cells alone), (\blacktriangle) Control (cells and paclitaxel at 28 μ M); n=3.

FIG. 34 is a graph which depicts proliferation of synovio-cytes at various concentrations of paclitaxel.

FIG. 35 is a bar graph which depicts the cytotoxicity of paclitaxel at various concentrations to proliferating synovio-cytes.

FIGS. 36A, 36B, and 36C are photographs of a series of gels which show the effect of various concentrations of paclitaxel on c-FOS expression.

FIGS. 37A and 37B are photographs of a series of gels which show the effect of various concentrations of paclitaxel on collagenase expression.

FIG. 38 is a bar graph which depicts the effects of paclitaxel on viability of normal chondrocytes in vitro.

FIG. 39 is a graph which shows the percentage of paclitaxel release based upon gelatinized-paclitaxel of either a large (7200 μ m) or small (2100 μ m) size.

FIG. 40 is a graph which shows the effect of gelatin and/or sodium chloride on the release of paclitaxel from PCL.

FIG. 41 is a graph which shows the release of paclitaxel from PDLLA-PEG-PDLLA cylinders containing 20% paclitaxel.

FIG. 42A is a graph which depicts the time course of paclitaxel release from 2.5 mg pellets of PCL. FIG. 42B is a graph which shows the percent paclitaxel remaining in the pellet, over time.

FIG. 43A is a graph which shows the effect of MePEG on paclitaxel release from PCL paste loaded with 20% paclitaxel. FIG. 43B is a graph which shows the percent paclitaxel remaining in the pellet, over time.

FIGS. 44A and 44B are graphs which show the effect of various concentrations of MePEG in PCL in terms of melting point (44A) and time to solidify (44B).

FIG. 45 is a graph which shows the effect of MePEG incorporation into PCL on the tensile strength and time to fail of the polymer.

FIG. 46 is a graph which shows the effect of irradiation on paclitaxel release.

FIGS. 47A, B, C, D and E show the effect of MTX release from PCL over time.

FIG. 48 is a graph of particle diameter (μ m) determined by a Coulter® LS130 Particle Size Analysis.

FIG. 49 is a graph of particle diameter (μ m) determined by a Coulter® LS130 Particle Size Analysis.

FIG. 50 is a graph which shows paclitaxel release from various polymeric formulations.

FIG. 51 is a graph which shows the effect of plasma opsonization of polymeric microspheres on the chemiluminescence response of neutrophils (20 mg/ml microspheres in 0.5 ml of cells (conc. 5×10^6 cells/ml) to PCL microspheres.

FIG. 52 is a graph which shows the effect of precoating plasma +/-2% pluronic F127 on the chemiluminescence response of neutrophils (5×10^6 cells/ml) to PCL microspheres.

FIG. 53 is a graph which shows the effect of precoating plasma +/-2% pluronic F127 on the chemiluminescence response of neutrophils (5×10^6 cells/ml) to PMMA microspheres.

FIG. 54 is a graph which shows the effect of precoating plasma +/-2% pluronic F127 on the chemiluminescence response of neutrophils (5×10^6 cells/ml) to PLA microspheres.

FIG. 55 is a graph which shows the effect of precoating plasma +/-2% pluronic F127 on the chemiluminescence response of neutrophils (5×10^6 cells/ml) to EVA:PLA microspheres.

FIG. 56 is a graph which shows the effect of precoating IgG (2 mg/ml), or 2% pluronic F127 then IgG (2 mg/ml) on the chemiluminescence response of neutrophils to PCL microspheres.

FIG. 57 is a graph which shows the effect of precoating IgG (2 mg/ml), or 2% pluronic F127 then IgG (2 mg/ml) on the chemiluminescence response of neutrophils to PMMA microspheres.

FIG. 58 is a graph which shows the effect of precoating IgG (2 mg/ml), or 2% pluronic F127 then IgG (2 mg/ml) on the chemiluminescence response of neutrophils to PVA microspheres.

FIG. 59 is a graph which shows the effect of precoating IgG (2 mg/ml), or 2% pluronic F127 then IgG (2 mg/ml) on the chemiluminescence response of neutrophils to EVA:PLA microspheres.

FIG. 60 is a photograph of 10% methotrexate ("MTX") loaded microspheres made from a 50:50 ratio of PLA:GA (TV 0.78).

FIG. 61 is a graph which depicts the release of 10% loaded vanadyl sulfate from PCL.

FIG. 62 is a photograph of hyaluronic acid microspheres containing vanadium sulfate.

FIG. 63A is a graph which depicts the release of organic vanadate from PCL. FIG. 63B depicts the percentage of organic vanadate remaining over a time course.

FIG. 64 is a photograph showing poly D,L lactic acid microspheres containing organic vanadate.

FIGS. 65A and 65B are photographs of control (uncoated) stents which show typical epithelial ingrowth seen at both 8 weeks (A) and at 16 weeks (B). Indentations of the stent tines (t) and narrowing of the lumen (lu) are shown. There is progressive epithelial overgrowth of the stent surface over this time by fibrous and inflammatory tissue.

FIGS. 66A, 66B, 66C, and 66D are a series of photographs which show control and paclitaxel-coated biliary stents. FIG. 66A illustrates the obliteration of the stent lumen by the process of benign epithelial overgrowth. At higher magnification (66B), the fibrous and inflammatory tissue is evident with little luminal space remaining. The paclitaxel-treated biliary duct remains patent (66C). At higher magnification, normal biliary tract epithelium is present with only minimal alteration of the mucosal lining by the coated stent tines (t).

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention provides methods and compositions which utilize anti-angiogenic factors. Briefly, within the context of the present invention, anti-angiogenic factors should be understood to include any protein, peptide, chemical, or other molecule, which acts to inhibit vascular growth. A variety of methods may be readily utilized to determine the anti-angiogenic activity of a given factor, including for example, chick chorioallantoic membrane ("CAM") assays. Briefly, as described in more detail below in Examples 2A and 2C, a portion of the shell from a freshly fertilized chicken egg is removed, and a methyl cellulose disk containing a sample of the anti-angiogenic factor to be tested is placed on the membrane. After several days (e.g., 48 hours), inhibition of vascular growth by the

sample to be tested may be readily determined by visualization of the chick chorioallantoic membrane in the region surrounding the methyl cellulose disk. Inhibition of vascular growth may also be determined quantitatively, for example, by determining the number and size of blood vessels surrounding the methyl cellulose disk, as compared to a control methyl cellulose disk. Although anti-angiogenic factors as described herein are considered to inhibit the formation of new blood vessels if they do so in merely a statistically significant manner, as compared to a control, within preferred aspects such anti-angiogenic factors will completely inhibit the formation of new blood vessels, as well as reduce the size and number of previously existing vessels.

In addition to the CAM assay described above, a variety of other assays may also be utilized to determine the efficacy of anti-angiogenic factors in vivo, including for example, mouse models which have been developed for this purpose (see Roberston et al., *Cancer Res.* 51:1339-1344, 1991). In addition, a variety of representative in vivo assays relating to various aspects of the inventions described herein have also been described in more detail below in Examples 5 to 7, and 17 to 19.

As noted above, the present invention provides compositions comprising an anti-angiogenic factor, and a polymeric carrier. Briefly, a wide variety of anti-angiogenic factors may be readily utilized within the context of the present invention. Representative examples include Anti-Invasive Factor, retinoic acid and derivatives thereof, paclitaxel, Suramin, Tissue Inhibitor of Metalloproteinase-1, Tissue Inhibitor of Metalloproteinase-2, Plasminogen Activator Inhibitor-1, Plasminogen Activator Inhibitor-2, and various forms of the lighter "d group" transition metals. These and other anti-angiogenic factors will be discussed in more detail below.

Briefly, Anti-Invasive Factor, or "AIF" which is prepared from extracts of cartilage, contains constituents which are responsible for inhibiting the growth of new blood vessels. These constituents comprise a family of 7 low molecular weight proteins (<50,000 daltons) (Kuettner and Pauli, "Inhibition of neovascularization by a cartilage factor" in *Development of the Vascular System*, Pitman Books (CIBA Foundation Symposium 100), pp. 163-173, 1983), including a variety of proteins which have inhibitory effects against a variety of proteases (Eisentein et al., *Am. J. Pathol.* 81:337-346, 1975; Langer et al., *Science* 193:70-72, 1976; and Horton et al., *Science* 199:1342-1345, 1978). AIF suitable for use within the present invention may be readily prepared utilizing techniques known in the art (e.g., Eisentein et al., supra; Kuettner and Pauli, supra; and Langer et al., supra). Purified constituents of AIF such as Cartilage-Derived Inhibitor ("CDI") (see Moses et al., *Science* 248:1408-1410, 1990) may also be readily prepared and utilized within the context of the present invention.

Retinoic acids alter the metabolism of extracellular matrix components, resulting in the inhibition of angiogenesis. Addition of proline analogs, angiostatic steroids, or heparin may be utilized in order to synergistically increase the anti-angiogenic effect of transretinoic acid. Retinoic acid, as well as derivatives thereof which may also be utilized in the context of the present invention, may be readily obtained from commercial sources, including for example, Sigma Chemical Co. (#R2625).

Paclitaxel is a highly derivatized diterpenoid (Wani et al., *J. Am. Chem. Soc.* 93:2325, 1971) which has been obtained from the harvested and dried bark of *Taxus brevifolia* (Pacific Yew.) and *Taxomyces Andreanae* and *Endophytic*

Fungus of the Pacific Yew (Stierle et al., *Science* 60:214-216, 1993). Generally, paclitaxel acts to stabilize microtubular structures by binding tubulin to form abnormal mitotic spindles. "Paclitaxel" (which should be understood herein to include analogues and derivatives such as, for example, TAXOL®, TAXOTERE®, 10-desacetyl analogues of paclitaxel and 3'-N-desbenzoyl-3'-N-t-butoxy carbonyl analogues of paclitaxel) may be readily prepared utilizing techniques known to those skilled in the art (see also WO 94/07882, WO 94/07881, WO 94/07880, WO 94/07876, WO 93/23555, WO 93/10076, U.S. Pat. Nos. 5,294,637, 5,283,253, 5,279,949, 5,274,137, 5,202,448, 5,200,534, 5,229,529, and EP 590267), or obtained from a variety of commercial sources, including for example, Sigma Chemical Co., St. Louis, Miss. (T7402—from *Taxus brevifolia*).

Suramin is a polysulfonated naphthylurea compound that is typically used as a trypanocidal agent. Briefly, Suramin blocks the specific cell surface binding of various growth factors such as platelet derived growth factor ("PDGF"), epidermal growth factor ("EGF"), transforming growth factor ("TGF-β"), insulin-like growth factor ("IGF-I"), and fibroblast growth factor ("βFGF"). Suramin may be prepared in accordance with known techniques, or readily obtained from a variety of commercial sources, including for example Mobay Chemical Co., New York. (see Gagliardi et al., *Cancer Res.* 52:5073-5075, 1992; and Coffey, Jr., et al., *J. of Cell. Phys.* 132:143-148, 1987).

Tissue Inhibitor of Metalloproteinases-1 ("TIMP") is secreted by endothelial cells which also secrete MTPases. TIMP is glycosylated and has a molecular weight of 28.5 kDa. TIMP-1 regulates angiogenesis by binding to activated metalloproteinases, thereby suppressing the invasion of blood vessels into the extracellular matrix. Tissue Inhibitor of Metalloproteinases-2 ("TIMP-2") may also be utilized to inhibit angiogenesis. Briefly, TIMP-2 is a 21 kDa nonglycosylated protein which binds to metalloproteinases in both the active and latent, proenzyme forms. Both TIMP-1 and TIMP-2 may be obtained from commercial sources such as Syngene, Boulder, Colo.

Plasminogen Activator Inhibitor-1 (PAI) is a 50 kDa glycoprotein which is present in blood platelets, and can also be synthesized by endothelial cells and muscle cells. PAI-1 inhibits t-PA and urokinase plasminogen activator at the basolateral site of the endothelium, and additionally regulates the fibrinolysis process. Plasminogen Activator Inhibitor-2 (PAI-2) is generally found only in the blood under certain circumstances such as in pregnancy, and in the presence of tumors. Briefly, PAI-2 is a 56 kDa protein which is secreted by monocytes and macrophages. It is believed to regulate fibrinolytic activity, and in particular inhibits urokinase plasminogen activator and tissue plasminogen activator, thereby preventing fibrinolysis.

Lighter "d group" transition metals include, for example, vanadium, molybdenum, tungsten, titanium, niobium, and tantalum species. Such transition metal species may form transition metal complexes. Suitable complexes of the above-mentioned transition metal species include oxo transition metal complexes.

Representative examples of vanadium complexes include oxo vanadium complexes such as vanadate and vanadyl complexes. Suitable vanadate complexes include metavanadate (i.e., VO_3^-) and orthovanadate (i.e., VO_4^{3-}) complexes such as, for example, ammonium metavanadate (i.e., NH_4VO_3), sodium metavanadate (i.e., NaVO_3), and sodium orthovanadate (i.e., Na_2VO_4). Suitable vanadyl (i.e., VO^{2+}) complexes include, for example, vanadyl acetylacetonate

and vanadyl sulfate including vanadyl sulfate hydrates such as vanadyl sulfate mono- and trihydrates.

Representative examples of tungsten and molybdenum complexes also include oxo complexes. Suitable oxo tungsten complexes include tungstate and tungsten oxide complexes. Suitable tungstate (i.e., WO_4^{2-}) complexes include ammonium tungstate (i.e., $(\text{NH}_4)_2\text{WO}_4$), calcium tungstate (i.e., CaWO_4), sodium tungstate dihydrate (i.e., $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$), and tungstic acid (i.e., H_2WO_4). Suitable tungsten oxides include tungsten (IV) oxide (i.e., WO_2) and tungsten (VI) oxide (i.e., WO_3). Suitable oxo molybdenum complexes include molybdate, molybdenum oxide, and molybdenyl complexes. Suitable molybdate (i.e., MoO_4^{2-}) complexes include ammonium molybdate (i.e., $(\text{NH}_4)_2\text{MoO}_4$) and its hydrates, sodium molybdate (i.e., Na_2MoO_4) and its hydrates, and potassium molybdate (i.e., K_2MoO_4) and its hydrates. Suitable molybdenum oxides include molybdenum (VI) oxide (i.e., MoO_3), molybdenum (VI) oxide (i.e., MoO_3), and molybdic acid. Suitable molybdenyl (i.e., MoO_2^{2+}) complexes include, for example, molybdenyl acetylacetonate. Other suitable tungsten and molybdenum complexes include hydroxo derivatives derived from, for example, glycerol, tartaric acid, and sugars.

A wide variety of other anti-angiogenic factors may also be utilized within the context of the present invention. Representative examples include Platelet Factor 4 (Sigma Chemical Co., #F1385); Protamine Sulphate (Clupeine) (Sigma Chemical Co., #P4505); Sulphated Chitin Derivatives (prepared from queen crab shells), (Sigma Chemical Co., #C3641; Murata et al., *Cancer Res.* 51:22-26, 1991); Sulphated Polysaccharide Peptidoglycan Complex (SP-PG) (the function of this compound may be enhanced by the presence of steroids such as estrogen, and tamoxifen citrate); Staurosporine (Sigma Chemical Co., #S4400); Modulators of Matrix Metabolism, including for example, proline analogs {[L-azetidine-2-carboxylic acid (LACA) (Sigma Chemical Co., #A0760)], cishydroxyproline, d,L-3,4-dehydroproline (Sigma Chemical Co., #D0265), Thiaprolone (Sigma Chemical Co., #T0631)], α,α -dipyridyl (Sigma Chemical Co., #D7505), β -aminopropionitrile fumarate (Sigma Chemical Co., #A3134)]}; MDL 27032 (4-propyl-5-(4-pyridinyl)-2(3H)-oxazolone; Merion Merrel Dow Research Institute); Methotrexate (Sigma Chemical Co., #A6770; Hirata et al., *Arthritis and Rheumatism* 32:1065-1073, 1989); Mitoxantrone (Polverini and Novak, *Biochem. Biophys. Res. Comm.* 140:901-907); Heparin (Folkman, *Bio. Phar.* 34:905-909, 1985; Sigma Chemical Co., #P8754); Interferons (e.g., Sigma Chemical Co., #13265); 2 Macroglobulin-serum (Sigma Chemical Co., #M7151); ChIMP-3 (Pavloff et al., *J. Bio. Chem.* 267:17321-17326, 1992); Chymostatin (Sigma Chemical Co., #C7268; Tomkinson et al., *Biochem J.* 286:475-480, 1992); β -Cyclodextrin Tetradecasulfate (Sigma Chemical Co., #C4767); Eponemycin; Camptothecin; Fumagillin (Sigma Chemical Co., #F6771; Canadian Patent No. 2,024,306; Ingber et al., *Nature* 348:555-557, 1990); Gold Sodium Thiomalate ("GST"; Sigma:G4022; Matsubara and Ziff, *J. Clin. Invest.* 79:1440-1446, 1987); (D-Penicillamine ("CDPT"; Sigma Chemical Co., #P4875 or P5000(HCl)); β -1-anticollagenase-serum; α 2-antiplasmin (Sigma Chem. Co.:A0914; Holmes et al., *J. Biol. Chem.* 262(4):1659-1664, 1987); Bisantrene (National Cancer Institute); Lobenzarit disodium (N-(2)-carboxyphenyl-4-chloroanthranilic acid disodium or "CCA"; Takeuchi et al., *Agents Actions* 36:312-316, 1992); Thalidomide; Angostatic steroid; AGM-1470; carboxynaminimidazole; metalloproteinase inhibitors such as BB94 and the peptide CDPGYIGSR-NH₂ (SEQUENCE ID NO. 1) (Iwaki Glass, Tokyo, Japan).

Although the above anti-angiogenic factors have been provided for the purposes of illustration, it should be understood that the present invention is not so limited. In particular, although certain anti-angiogenic factors are specifically referred to above, the present invention should be understood to include analogues, derivatives and conjugates of such anti-angiogenic factors. For example, paclitaxel should be understood to refer to not only the common chemically available form of paclitaxel, but analogues (e.g., taxotere, as noted above) and paclitaxel conjugates (e.g., paclitaxel-PEG, paclitaxel-dextran, or paclitaxel-xylos).

Anti-angiogenic compositions of the present invention may additionally comprise a wide variety of compounds in addition to the anti-angiogenic factor and polymeric carrier. For example, anti-angiogenic compositions of the present invention may also, within certain embodiments of the invention, also comprise one or more antibiotics, anti-inflammatories, anti-vital agents, anti-fungal agents and/or anti-protozoal agents. Representative examples of antibiotics included within the compositions described herein include: penicillins; cephalosporins such as cefadroxil, cefazolin, cefaclor; aminoglycosides such as gentamycin and tobramycin; sulfonamides such as sulfamethoxazole; and metronidazole. Representative examples of anti-inflammatories include: steroids such as prednisone, prednisolone, hydrocortisone, adrenocorticotrophic hormone, and sulfasalazine; and non-steroidal anti-inflammatory drugs ("NSAIDS") such as aspirin, ibuprofen, naproxen, fenoprofen, indomethacin, and phenylbutazone. Representative examples of antiviral agents include acyclovir, ganciclovir, zidovudine. Representative examples of anti-fungal agents include: nystatin, ketoconazole, griseofulvin, flucytosine, miconazole, clotrimazole. Representative examples of antiprotozoal agents include: pentamidine isethionate, quinine, chloroquine, and mefloquine.

Anti-angiogenic compositions of the present invention may also contain one or more hormones such as thyroid hormone, estrogen, progesterone, cortisone and/or growth hormone, other biologically active molecules such as insulin, as well as T_H1 (e.g., Interleukins-2, -12, and -15, gamma interferon) or T_H2 (e.g., Interleukins-4 and -10) cytokines.

Within certain preferred embodiments of the invention, anti-angiogenic compositions are provided which contain one or more compounds which disrupt microtubule function. Representative examples of such compounds include paclitaxel (discussed above), estramustine (available from Sigma; Wang and Stearns *Cancer Res.* 48:6262-6271, 1988), epothilone, curacin-A, colchicine, methotrexate, vinblastine and 4-tert-butyl-[3-(2-chloroethyl)ureido]benzene ("tBCEU").

Anti-angiogenic compositions of the present invention may also contain a wide variety of other compounds, including for example: α -adrenergic blocking agents, angiotensin II receptor antagonists and receptor antagonists for histamine, serotonin, endothelin; inhibitors of the sodium/hydrogen antiporter (e.g., amiloride and its derivatives); agents that modulate intracellular Ca^{2+} transport such as L-type (e.g., diltiazem, nifedipine, verapamil) or T-type Ca^{2+} channel blockers (e.g., amiloride), calmodulin antagonists (e.g., H_7) and inhibitors of the sodium/calcium antiporter (e.g., amiloride); ap-1 inhibitors (for tyrosine kinases, protein kinase C, myosin light chain kinase, Ca^{2+} /calmodulin kinase II, casein kinase II); anti-depressants (e.g., amitriptyline, fluoxetine, LUVOX® and FAXIL®); cytokine and/or growth factors, as well as their respective receptors, (e.g., the interleukins, α , β or γ -IFN, GM-CSF,

G-CSF, epidermal growth factor, transforming growth factors alpha and beta, TNF, and antagonists of vascular epithelial growth factor, endothelial growth factor, acidic or basic fibroblast growth factors, and platelet derived growth factor); inhibitors of the IP_3 receptor (e.g., heparin); protease and collagenase inhibitors (e.g., TIMPs, discussed above); nitrovasodilators (e.g., isosorbide dinitrate); anti-mitotic agents (e.g., colchicine, anthracyclines and other antibiotics, folate antagonists and other anti-metabolites, vinca alkaloids, nitrosoureas, DNA alkylating agents, topoisomerase inhibitors, purine antagonists and analogs, pyrimidine antagonists and analogs, alkyl sulfonates); immunosuppressive agents (e.g., adrenocorticosteroids, cyclosporine); sense or antisense oligonucleotides (e.g., DNA, RNA, nucleic acid analogues (e.g., peptide nucleic acids) or any combinations of these); and inhibitors of transcription factor activity (e.g., lighter d group transition metals).

Anti-angiogenic compositions of the present invention may also comprise additional ingredients such as surfactants (either hydrophilic or hydrophobic; see Example 13), antineoplastic or chemotherapeutic agents (e.g., 5-fluorouracil, vincristine, vinblastine, cisplatin, doxorubicin, adriamycin, or tamoxifen), radioactive agents (e.g., Cu-64, Ca-67, Ga-68, Zr-89, Ku-97, Tc-99m, Rh-105, Pd-109, In-111, I-123, I-125, I-131, Re-186, Re-188, Au-198, Au-199, Pb-203, At-211, Pb-212 and Bi-212) or toxins (e.g., ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pokeweed antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A).

As noted above, anti-angiogenic compositions of the present invention comprise an anti-angiogenic factor and a polymeric carrier. In addition to the wide array of anti-angiogenic factors and other compounds discussed above, anti-angiogenic compositions of the present invention are provided in a wide variety of polymeric carriers, including for example both biodegradable and non-biodegradable compositions. Representative examples of biodegradable compositions include albumin, gelatin, starch, cellulose, dextrans, polysaccharides, fibrinogen, poly (DL lactide), poly (DL-lactide-co-glycolide), poly (glycolide), poly (hydroxybutyrate), poly (alkylcarbonate) and poly (orthoesters) (see generally, Illum, L., Davids, S. S. (eds.) "Polymers in controlled Drug Delivery" Wright, Bristol, 1987; Arshady, J. *Controlled Release* 17:1-22, 1991; Pitt, Int. J. Phar. 59:173-196, 1990; Holland et al., *J. Controlled Release* 4:155-0180, 1986). Representative examples of nondegradable polymers include EVA copolymers, silicone rubber and poly (methylmethacrylate). Particularly preferred polymeric carriers include poly (ethylene-vinyl acetate)(40% cross-linked), poly (DL-lactic acid) oligomers and polymers, poly (L-lactic acid) oligomers and polymers, poly (glycolic acid), copolymers of lactic acid and glycolic acid, poly (caprolactone), poly (valerolactone), polyanhydrides, copolymers of poly (caprolactone) or poly (lactic acid) with polyethylene glycol and blends thereof.

Polymeric carriers may be fashioned in a variety of forms, including for example, rod-shaped devices, pellets, slabs, or capsules (see, e.g., Goodell et al., *Am. J. Hosp. Pharm.* 43:1454-1461, 1986; Langer et al., "Controlled release of macromolecules from polymers", in *Biomedical polymers. Polymeric materials and pharmaceuticals for biomedical use*, Goldberg, E. P., Nakagim, A. (eds.) Academic Press, pp. 113-137, 1980; Rhine et al., *J. Pharm. Sci.* 69:265-270, 1980; Brown et al., *J. Pharm. Sci.* 72:1181-1185, 1983; and Bawa et al., *J. Controlled Release* 1:259-267, 1985). Anti-angiogenic factors may be linked by occlusion in the matri-

ces of the polymer, bound by covalent linkages, or encapsulated in microcapsules. Within certain preferred embodiments of the invention, anti-angiogenic compositions are provided in non-capsular formulations such as microspheres (ranging from nanometers to micrometers in size), pastes, threads of various size, films and sprays.

Preferably, anti-angiogenic compositions of the present invention (which comprise one or more anti-angiogenic factors, and a polymeric carrier) are fashioned in a manner appropriate to the intended use. Within certain aspects of the present invention, the anti-angiogenic composition should be biocompatible, and release one or more anti-angiogenic factors over a period of several days to months. For example, "quick release" or "burst" anti-angiogenic compositions are provided that release greater than 10%, 20%, or 25% (w/v) of an anti-angiogenic factor (e.g., paclitaxel) over a period of 7 to 10 days. Such "quick release" compositions should, within certain embodiments, be capable of releasing chemotherapeutic levels (where applicable) of a desired anti-angiogenic factor. Within other embodiments, "low release" anti-angiogenic compositions are provided that release less than 1% (w/v) of an anti-angiogenic factor over a period of 7 to 10 days. Further, anti-angiogenic compositions of the present invention should preferably be stable for several months and capable of being produced and maintained under sterile conditions.

Within certain aspects of the present invention, anti-angiogenic compositions may be fashioned in any size ranging from 50 nm to 500 μ m, depending upon the particular use. For example, when used for the purpose of tumor embolization (as discussed below), it is generally preferable to fashion the anti-angiogenic composition in microspheres of between 15 and 500 μ m, preferably between 15 and 200 μ m, and most preferably, between 25 and 150 μ m. Alternatively, such compositions may also be readily applied as a "spray", which solidifies into a film or coating. Such sprays may be prepared from microspheres of a wide array of sizes, including for example, from 0.1 μ m to 3 μ m, from 10 μ m to 30 μ m, and from 30 μ m to 100 μ m (see Example 8).

Anti-angiogenic compositions may also be prepared, given the disclosure provided herein, for a variety of other applications. For example, for administration to the cornea, the anti-angiogenic factors of the present invention may be incorporated into muco-adhesive polymers (e.g., polyacrylic acids such as (CARBOPOL®), dextran, polymethacrylate, or starch (see LeYung and Robinson, *J. of Controlled Rel.* 5:223, 1988)), or nanometer-sized microspheres (see generally, Kreuter *J. Controlled Release* 16:169-176, 1991; Couvreur and Vauthier, *J. Controlled Release* 17:187-198, 1991).

Anti-angiogenic compositions of the present invention may also be prepared in a variety of "paste" or gel forms. For example, within one embodiment of the invention, anti-angiogenic compositions are provided which are liquid at one temperature (e.g., temperature greater than 37° C., such as 40° C., 45° C., 50° C., 55° C. or 60° C.), and solid or semi-solid at another temperature (e.g., ambient body temperature, or any temperature lower than 37° C.). Such "thermopastes" may be readily made given the disclosure provided herein (see, e.g., Examples 10 and 14).

Within yet other aspects of the invention, the anti-angiogenic compositions of the present invention may be formed as a film. Preferably, such films are generally less than 5, 4, 3, 2, or 1, mm thick, more preferably less than 0.75 mm or 0.5 mm thick, and most preferably less than 500 μ m

to 100 μ m thick. Such films are preferably flexible with a good tensile strength (e.g., greater than 50, preferably greater than 100, and more preferably greater than 150 or 200N/cm²), good adhesive properties (i.e., readily adheres to moist or wet surfaces), and has controlled permeability. Representative examples of such films are set forth below in the Examples (see e.g., Example 13).

Representative examples of the incorporation of anti-angiogenic factors such as those described above into a polymeric carriers is described in more detail below in Examples 3, 4 and 8-15.

POLYMERIC CARRIERS FOR THE RELEASE OF HYDROPHOBIC COMPOUNDS

Within further aspects of the present invention, polymeric carriers are provided which are adapted to contain and release a hydrophobic compound, the carrier containing the hydrophobic compound in combination with a carbohydrate, protein or polypeptide. Within certain embodiments, the polymeric carrier contains or comprises regions, pockets, or granules of one or more hydrophobic compounds. For example, within one embodiment of the invention, hydrophobic compounds may be incorporated within a matrix which contains the hydrophobic compound, followed by incorporation of the matrix within the polymeric carrier. A variety of matrices can be utilized in this regard, including for example, carbohydrates and polysaccharides such as starch, cellulose, dextran, methylcellulose, and hyaluronic acid, proteins or polypeptides such as albumin, collagen and gelatin (see e.g., Example 31). Within alternative embodiments, hydrophobic compounds may be contained within a hydrophobic core, and this core contained within a hydrophilic shell. For example, as described in Example 38, paclitaxel may be incorporated into a hydrophobic core (e.g., of the poly D,L lactic acid-PEG or MePEG aggregate) which has a hydrophilic shell.

A wide variety of hydrophobic compounds may be released from the polymeric carriers described above, including for example: certain hydrophobic compounds which disrupt microtubule function such as paclitaxel and estramustine; hydrophobic proteins such as myelin basic protein, proteolipid proteins of CNS myelin, hydrophobic cell wall protein, porins, membrane proteins (*EMBO J.* 12(9):3409-3415, 1993), myelin oligodendrocyte glycoprotein ("MOG") (*Biochem. and Mol. Biol. Int.* 30(5):945-958, 1993, P27 *Cancer Res.* 53(17):4096-4101, 1993), bacterioopsin, human surfactant protein ("HSB"; *J. Biol. Chem.* 268(15):11160-11166, 1993), and SP-B or SP-C (*Biochimica et Biophysica Acta* 1105(1):161-169, 1992).

ARTERIAL EMBOLIZATION

In addition to the compositions described above, the present invention also provides a variety of methods which utilize the above-described anti-angiogenic compositions. In particular, within one aspect of the present invention methods are provided for embolizing a blood vessel, comprising the step of delivering into the vessel a therapeutically effective amount of an anti-angiogenic composition (as described above), such that the blood vessel is effectively occluded. Therapeutically effective amounts suitable for occluding blood vessels may be readily determined given the disclosure provided below, and as described in Example 6. Within a particularly preferred embodiment, the anti-angiogenic composition is delivered to a blood vessel which nourishes a tumor (see FIG. 13).

Briefly, there are a number of clinical situations (e.g., bleeding, tumor development) where it is desirable to reduce

or abolish the blood supply to an organ or region. As described in greater detail below, this may be accomplished by injecting anti-angiogenic compositions of the present invention into a desired blood vessel through a selectively positioned catheter (see FIG. 13). The composition travels via the blood stream until it becomes wedged in the vasculature, thereby physically (or chemically) occluding the blood vessel. The reduced or abolished blood flow to the selected area results in infarction (cell death due to an inadequate supply of oxygen and nutrients) or reduced blood loss from a damaged vessel.

For use in embolization therapy, anti-angiogenic compositions of the present invention are preferably non-toxic, thrombogenic, easy to inject down vascular catheters, radio-opaque, rapid and permanent in effect, sterile, and readily available in different shapes or sizes at the time of the procedure. In addition, the compositions preferably result in the slow (ideally, over a period of several weeks to months) release of an anti-angiogenic factor. Particularly preferred anti-angiogenic compositions should have a predictable size of 15–200 μm after being injected into the vascular system. Preferably, they should not clump into larger particles either in solution or once injected. In addition, preferable compositions should not change shape or physical properties during storage prior to use.

Embolization therapy may be utilized in at least three principal ways to assist in the management of neoplasms: (1) definitive treatment of tumors (usually benign); (2) for preoperative embolization; and (3) for palliative embolization. Briefly, benign tumors may sometimes be successfully treated by embolization therapy alone. Examples of such tumors include simple tumors of vascular origin (e.g., haemangiomas), endocrine tumors such as parathyroid adenomas, and benign bone tumors.

For other tumors, (e.g., renal adenocarcinoma), preoperative embolization may be employed hours or days before surgical resection in order to reduce operative blood loss, shorten the duration of the operation, and reduce the risk of dissemination of viable malignant cells by surgical manipulation of the tumor. Many tumors may be successfully embolized preoperatively, including for example nasopharyngeal tumors, glomus jugular tumors, meningiomas, chemodectomas, and vagal neuromas.

Embolization may also be utilized as a primary mode of treatment for inoperable malignancies, in order to extend the survival time of patients with advanced disease. Embolization may produce a marked improvement in the quality of life of patients with malignant tumors by alleviating unpleasant symptoms such as bleeding, venous obstruction and tracheal compression. The greatest benefit from palliative tumor embolization, however, may be seen in patients suffering from the humoral effects of malignant endocrine tumors, wherein metastases from carcinoid tumors and other endocrine neoplasms such as insulinomas and glucagonomas may be slow growing, and yet cause great distress by virtue of the endocrine syndromes which they produce.

In general, embolization therapy utilizing anti-angiogenic compositions of the present invention is typically performed in a similar manner, regardless of the site. Briefly, angiography (a road map of the blood vessels) of the area to be embolized is first performed by injecting radiopaque contrast through a catheter inserted into an artery or vein (depending on the site to be embolized) as an X-ray is taken. The catheter may be inserted either percutaneously or by surgery. The blood vessel is then embolized by refluxing anti-angiogenic compositions of the present invention

through the catheter, until flow is observed to cease. Occlusion may be confirmed by repeating the angiogram.

Embolization therapy generally results in the distribution of compositions containing anti-angiogenic factors throughout the interstices of the tumor or vascular mass to be treated. The physical bulk of the embolic particles clogging the arterial lumen results in the occlusion of the blood supply. In addition to this effect, the presence of an anti-angiogenic factor(s) prevents the formation of new blood vessels to supply the tumor or vascular mass, enhancing the devitalizing effect of cutting off the blood supply.

Therefore, it should be evident that a wide variety of tumors may be embolized utilizing the compositions of the present invention. Briefly, tumors are typically divided into two classes: benign and malignant. In a benign tumor the cells retain their differentiated features and do not divide in a completely uncontrolled manner. In addition, the tumor is localized and nonmetastatic. In a malignant tumor, the cells become undifferentiated, do not respond to the body's growth and hormonal signals, and multiply in an uncontrolled manner; the tumor is invasive and capable of spreading to distant sites (metastasizing).

Within one aspect of the present invention, metastases (secondary tumors) of the liver may be treated utilizing embolization therapy. Briefly, a catheter is inserted via the femoral or brachial artery and advanced into the hepatic artery by steering it through the arterial system under fluoroscopic guidance. The catheter is advanced into the hepatic arterial tree as far as necessary to allow complete blockage of the blood vessels supplying the tumor(s), while sparing as many of the arterial branches supplying normal structures as possible. Ideally this will be a segmental branch of the hepatic artery, but it could be that the entire hepatic artery distal to the origin of the gastroduodenal artery, or even multiple separate arteries, will need to be blocked depending on the extent of tumor and its individual blood supply. Once the desired catheter position is achieved, the artery is embolized by injecting anti-angiogenic compositions (as described above) through the arterial catheter until flow in the artery to be blocked ceases, preferably even after observation for 5 minutes. Occlusion of the artery may be confirmed by injecting radiopaque contrast through the catheter and demonstrating by fluoroscopy or X-ray film that the vessel which previously filled with contrast no longer does so. The same procedure may be repeated with each feeding artery to be occluded.

As noted above, both benign and malignant tumors may be embolized utilizing compositions of the present invention. Representative examples of benign hepatic tumors include Hepatocellular Adenoma, Cavernous Haemangioma, and Focal Nodular Hyperplasia. Other benign tumors, which are more rare and often do not have clinical manifestations, may also be treated. These include Bile Duct Adenomas, Bile Duct Cystadenomas, Fibromas, Lipomas, Leiomyomas, Mesotheliomas, Teratomas, Myxomas, and Nodular Regenerative Hyperplasia.

Malignant Hepatic Tumors are generally subdivided into two categories: primary and secondary. Primary tumors arise directly from the tissue in which they are found. Thus, a primary liver tumor is derived originally from the cells which make up the liver tissue (such as hepatocytes and biliary cells). Representative examples of primary hepatic malignancies which may be treated by arterial embolization include Hepatocellular carcinoma, Cholangiocarcinoma, Angiosarcoma, Cystadenocarcinoma, Squamous Cell Carcinoma, and Hepatoblastoma.

A secondary tumor, or metastasis, is a tumor which originated elsewhere in the body but has now spread to a distant organ. The common routes for metastasis are direct growth into adjacent structures, spread through the vascular or lymphatic systems, and tracking along tissue planes and body spaces (peritoneal fluid, cerebrospinal fluid, etc.). Secondary hepatic tumors are one of the most common causes of death in the cancer patient and are by far and away the most common form of liver tumor. Although virtually any malignancy can metastasize to the liver, tumors which are most likely to spread to the liver include: cancer of the stomach, colon, and pancreas; melanoma; tumors of the lung, oropharynx, and bladder; Hodgkin's and non-Hodgkin's lymphoma; tumors of the breast, ovary, and prostate. Each one of the above-named primary tumors has numerous different tumor types which may be treated by arterial embolization (for example, there are over 32 different types of ovarian cancer).

As noted above, embolization therapy utilizing anti-angiogenic compositions of the present invention may also be applied to a variety of other clinical situations where it is desired to occlude blood vessels. Within one aspect of the present invention, arteriovenous malformation may be treated by administration of one of the above-described compositions. Briefly, arteriovenous malformations (vascular malformations) refers to a group of diseases wherein at least one (and most typically, many) abnormal communications between arteries and veins occur, resulting in a local tumor-like mass composed predominantly of blood vessels. Such disease may be either congenital or acquired.

Within one embodiment of the invention, an arteriovenous malformation may be treated by inserting a catheter via the femoral or brachial artery, and advancing it into the feeding artery under fluoroscopic guidance. The catheter is preferably advanced as far as necessary to allow complete blockage of the blood vessels supplying the vascular malformation, while sparing as many of the arterial branches supplying normal structures as possible (ideally this will be a single artery, but most often multiple separate arteries may need to be occluded, depending on the extent of the vascular malformation and its individual blood supply). Once the desired catheter position is achieved, each artery may be embolized utilizing anti-angiogenic compositions of the present invention.

Within another aspect of the invention, embolization may be accomplished in order to treat conditions of excessive bleeding. For example, menorrhagia (excessive bleeding with menstruation) may be readily treated by embolization of uterine arteries. Briefly, the uterine arteries are branches of the internal iliac arteries bilaterally. Within one embodiment of the invention, a catheter may be inserted via the femoral or brachial artery, and advanced into each uterine artery by steering it through the arterial system under fluoroscopic guidance. The catheter should be advanced as far as necessary to allow complete blockage of the blood vessels to the uterus, while sparing as many arterial branches that arise from the uterine artery and supply normal structures as possible. Ideally a single uterine artery on each side may be embolized, but occasionally multiple separate arteries may need to be blocked depending on the individual blood supply. Once the desired catheter position is achieved, each artery may be embolized by administration of the anti-angiogenic compositions as described above.

In a like manner, arterial embolization may be accomplished in a variety of other conditions, including for example, for acute bleeding, vascular abnormalities, central nervous system disorders, and hypersplenism.

USE OF ANTI-ANGIOGENIC COMPOSITIONS AS COATINGS FOR STENTS

As noted above, the present invention also provides stents, comprising a generally tubular structure (which includes for example, spiral shapes), the surface of which is coated with a composition as described above. Briefly, a stent is a scaffolding, usually cylindrical in shape, that may be inserted into a body passageway (e.g., bile ducts) or a portion of a body passageway, which has been narrowed, irregularly contoured, obstructed, or occluded by a disease process (e.g., ingrowth by a tumor) in order to prevent closure or reclosure of the passageway. Stents act by physically holding open the walls of the body passage into which they are inserted.

A variety of stents may be utilized within the context of the present invention, including for example, esophageal stents, vascular stents, biliary stents, pancreatic stents, ureteric and urethral stents, lacrimal stents, Eustachian tube stents, fallopian tube stents and tracheal/bronchial stents.

Stents may be readily obtained from commercial sources, or constructed in accordance with well-known techniques. Representative examples of stents include those described in U.S. Pat. No. 4,768,523, entitled "Hydrogel Adhesive;" U.S. Pat. No. 4,776,337, entitled "Expandable Intraluminal Graft, and Method and Apparatus for Implanting and Expandable Intraluminal Graft;" U.S. Pat. No. 5,041,126 entitled "Endovascular Stent and Delivery System;" U.S. Pat. No. 5,052,998 entitled "Indwelling Stent and Method of Use;" U.S. Pat. No. 5,064,435 entitled "Self-Expanding Prosthesis Having Stable Axial Length;" U.S. Pat. No. 5,089,606, entitled "Water-insoluble Polysaccharide Hydrogel Foam for Medical Applications;" U.S. Pat. No. 5,147,370, entitled "Nitinol Stent for Hollow Body Conduits;" U.S. Pat. No. 5,176,626, entitled "Indwelling Stent;" U.S. Pat. No. 5,213,580, entitled "Biodegradable polymeric Endoluminal Sealing Process;" and U.S. Pat. No. 5,328,471, entitled "Method and Apparatus for Treatment of Focal Disease in Hollow Tubular Organs and Other Tissue Lumens."

Stents may be coated with anti-angiogenic compositions or anti-angiogenic factors of the present invention in a variety of manners, including for example: (a) by directly affixing to the stent an anti-angiogenic composition (e.g., by either spraying the stent with a polymer/drug film, or by dipping the stent into a polymer/drug solution), (b) by coating the stent with a substance such as a hydrogel which will in turn absorb the anti-angiogenic composition (or anti-angiogenic factor above), (c) by interweaving anti-angiogenic composition coated thread (or the polymer itself formed into a thread) into the stent structure, (d) by inserting the stent into a sleeve or mesh which is comprised of or coated with an anti-angiogenic composition, or (e) constructing the stent itself with an anti-angiogenic composition. Within preferred embodiments of the invention, the composition should firmly adhere to the stent during storage and at the time of insertion, and should not be dislodged from the stent when the diameter is expanded from its collapsed size to its full expansion size. The anti-angiogenic composition should also preferably not degrade during storage, prior to insertion, or when warmed to body temperature after expansion inside the body. In addition, it should preferably coat the stent smoothly and evenly, with a uniform distribution of angiogenesis inhibitor, while not changing the stent contour. Within preferred embodiments of the invention, the anti-angiogenic composition should provide a uniform, predictable, prolonged release of the anti-angiogenic factor into the tissue surrounding the stent

once it has been deployed. For vascular stents, in addition to the above properties, the composition should not render the stent thrombogenic (causing blood clots to form), or cause significant turbulence in blood flow (more than the stent itself would be expected to cause if it was uncoated).

Within another aspect of the present invention, methods are provided for expanding the lumen of a body passageway, comprising inserting a stent into the passageway, the stent having a generally tubular structure, the surface of the structure being coated with an anti-angiogenic composition (or, an antioangiogenic factor alone), such that the passageway is expanded. A variety of embodiments are described below wherein the lumen of a body passageway is expanded in order to eliminate a biliary, esophageal, tracheal/bronchial, urethral or vascular obstruction. In addition, a representative example is described in more detail below in Example 7.

Generally, stents are inserted in a similar fashion regardless of the site or the disease being treated. Briefly, a preinsertion examination, usually a diagnostic imaging procedure, endoscopy, or direct visualization at the time of surgery, is generally first performed in order to determine the appropriate positioning for stent insertion. A guidewire is then advanced through the lesion or proposed site of insertion, and over this is passed a delivery catheter which allows a stent in its collapsed form to be inserted. Typically, stents are capable of being compressed, so that they can be inserted through tiny cavities via small catheters, and then expanded to a larger diameter once they are at the desired location. Once expanded, the stent physically forces the walls of the passageway apart and holds them open. As such, they are capable of insertion via a small opening, and yet are still able to hold open a large diameter cavity or passageway. The stent may be self-expanding (e.g., the Wallstent and Gianturco stents), balloon expandable (e.g., the Palmaz stent and Strecker stent), or implanted by a change in temperature (e.g., the Nitinol stent).

Stents are typically maneuvered into place under radiologic or direct visual control, taking particular care to place the stent precisely across the narrowing in the organ being treated. The delivery catheter is then removed, leaving the stent standing on its own as a scaffold. A post insertion examination, usually an x-ray, is often utilized to confirm appropriate positioning.

Within a preferred embodiment of the invention, methods are provided for eliminating biliary obstructions, comprising inserting a biliary stent into a biliary passageway, the stent having a generally tubular structure, the surface of the structure being coated with a composition as described above, such that the biliary obstruction is eliminated. Briefly, tumor overgrowth of the common bile duct results in progressive cholestatic jaundice which is incompatible with life. Generally, the biliary system which drains bile from the liver into the duodenum is most often obstructed by (1) a tumor composed of bile duct cells (cholangiocarcinoma), (2) a tumor which invades the bile duct (e.g., pancreatic carcinoma), or (3) a tumor which exerts extrinsic pressure and compresses the bile duct (e.g., enlarged lymph nodes).

Both primary biliary tumors, as well as other tumors which cause compression of the biliary tree may be treated utilizing the stents described herein. One example of primary biliary tumors are adenocarcinomas (which are also called Klatskin tumors when found at the bifurcation of the common hepatic duct). These tumors are also referred to as biliary carcinomas, choledocholangiocarcinomas, or adenocarcinomas of the biliary system. Benign tumors which

affect the bile duct (e.g., adenoma of the biliary system), and, in rare cases, squamous cell carcinomas of the bile duct and adenocarcinomas of the gallbladder, may also cause compression of the biliary tree and therefore, result in biliary obstruction.

Compression of the biliary tree is most commonly due to tumors of the liver and pancreas which compress and therefore obstruct the ducts. Most of the tumors from the pancreas arise from cells of the pancreatic ducts. This is a highly fatal form of cancer (5% of all cancer deaths; 26,000 new cases per year in the U.S.) with an average of 6 months survival and a 1 year survival rate of only 10%. When these tumors are located in the head of the pancreas they frequently cause biliary obstruction, and this detracts significantly from the quality of life of the patient. While all types of pancreatic tumors are generally referred to as "carcinoma of the pancreas" there are histologic subtypes including: adenocarcinoma, adenosquamous carcinoma, cystadenocarcinoma, and acinar cell carcinoma. Hepatic tumors, as discussed above, may also cause compression of the biliary tree, and therefore cause obstruction of the biliary ducts.

Within one embodiment of the invention, a biliary stent is first inserted into a biliary passageway in one of several ways: from the top end by inserting a needle through the abdominal wall and through the liver (a percutaneous transhepatic cholangiogram or "PTC"); from the bottom end by cannulating the bile duct through an endoscope inserted through the mouth, stomach, and duodenum (an endoscopic retrograde cholangiogram or "ERCP"); or by direct incision during a surgical procedure. A preinsertion examination, PTC, ERCP, or direct visualization at the time of surgery should generally be performed to determine the appropriate position for stent insertion. A guidewire is then advanced through the lesion, and over this a delivery catheter is passed to allow the stent to be inserted in its collapsed form. If the diagnostic exam was a PTC, the guidewire and delivery catheter is inserted via the abdominal wall, while if the original exam was an ERCP the stent may be placed via the mouth. The stent is then positioned under radiologic, endoscopic, or direct visual control taking particular care to place it precisely across the narrowing in the bile duct. The delivery catheter is then removed leaving the stent standing as a scaffolding which holds the bile duct open. A further cholangiogram may be performed to document that the stent is appropriately positioned.

Within yet another embodiment of the invention, methods are provided for eliminating esophageal obstructions, comprising inserting an esophageal stent into an esophagus, the stent having a generally tubular structure, the surface of the structure being coated with an anti-angiogenic composition as described above, such that the esophageal obstruction is eliminated. Briefly, the esophagus is the hollow tube which transports food and liquids from the mouth to the stomach. Cancer of the esophagus or invasion by cancer arising in adjacent organs (e.g., cancer of the stomach or lung) results in the inability to swallow food or saliva. Within this embodiment, a preinsertion examination, usually a barium swallow or endoscopy should generally be performed in order to determine the appropriate position for stent insertion. A catheter or endoscope may then be positioned through the mouth, and a guidewire is advanced through the blockage. A stent delivery catheter is passed over the guidewire under radiologic or endoscopic control, and a stent is placed precisely across the narrowing in the esophagus. A post insertion examination, usually a barium swallow x-ray, may be utilized to confirm appropriate positioning.

Within other embodiments of the invention, methods are provided for eliminating tracheal/bronchial obstructions,

comprising inserting a tracheal/bronchial stent into the trachea or bronchi, the stent having a generally tubular structure, the surface of which is coated with an anti-angiogenic composition as described above, such that the tracheal/bronchial obstruction is eliminated. Briefly, the trachea and bronchi are tubes which carry air from the mouth and nose to the lungs. Blockage of the trachea by cancer, invasion by cancer arising in adjacent organs (e.g., cancer of the lung), or collapse of the trachea or bronchi due to chondromalacia (weakening of the cartilage rings) results in inability to breathe. Within this embodiment of the invention, preinsertion examination, usually an endoscopy, should generally be performed in order to determine the appropriate position for stent insertion. A catheter or endoscope is then positioned through the mouth, and a guidewire advanced through the blockage. A delivery catheter is then passed over the guidewire in order to allow a collapsed stent to be inserted. The stent is placed under radiologic or endoscopic control in order to place it precisely across the narrowing. The delivery catheter may then be removed leaving the stent standing as a scaffold on its own. A post insertion examination, usually a bronchoscopy may be utilized to confirm appropriate positioning.

Within another embodiment of the invention, methods are provided for eliminating urethral obstructions, comprising inserting a urethral stent into a urethra, the stent having a generally tubular structure, the surface of the structure being coated with an anti-angiogenic composition as described above, such that the urethral obstruction is eliminated. Briefly, the urethra is the tube which drains the bladder through the penis. Extrinsic narrowing of the urethra as it passes through the prostate, due to hypertrophy of the prostate, occurs in virtually every man over the age of 60 and causes progressive difficulty with urination. Within this embodiment, a preinsertion examination, usually an endoscopy or urethrogram should generally first be performed in order to determine the appropriate position for stent insertion, which is above the external urinary sphincter at the lower end, and close to flush with the bladder neck at the upper end. An endoscope or catheter is then positioned through the penile opening and a guidewire advanced into the bladder. A delivery catheter is then passed over the guidewire in order to allow stent insertion. The delivery catheter is then removed, and the stent expanded into place. A post insertion examination, usually endoscopy or retrograde urethrogram, may be utilized to confirm appropriate position.

Within another embodiment of the invention, methods are provided for eliminating vascular obstructions, comprising inserting a vascular stent into a blood vessel, the stent having a generally tubular structure, the surface of the structure being coated with an anti-angiogenic composition as described above, such that the vascular obstruction is eliminated. Briefly, stents may be placed in a wide array of blood vessels, both arteries and veins, to prevent recurrent stenosis at the site of failed angioplasties, to treat narrowings that would likely fail if treated with angioplasty, and to treat post surgical narrowings (e.g., dialysis graft stenosis). Representative examples of suitable sites include the iliac, renal, and coronary arteries, the superior vena cava, and in dialysis grafts. Within one embodiment, angiography is first performed in order to localize the site for placement of the stent. This is typically accomplished by injecting radiopaque contrast through a catheter inserted into an artery or vein as an x-ray is taken. A catheter may then be inserted either percutaneously or by surgery into the femoral artery, brachial artery, femoral vein, or brachial vein, and advanced

into the appropriate blood vessel by steering it through the vascular system under fluoroscopic guidance. A stent may then be positioned across the vascular stenosis. A post insertion angiogram may also be utilized in order to confirm appropriate positioning.

USE OF ANTI-ANGIOGENIC COMPOSITIONS IN SURGICAL PROCEDURES

As noted above, anti-angiogenic compositions may be utilized in a wide variety of surgical procedures. For example, within one aspect of the present invention an anti-angiogenic compositions (in the form of, for example, a spray or film) may be utilized to coat or spray an area prior to removal of a tumor, in order to isolate normal surrounding tissues from malignant tissue, and/or to prevent the spread of disease to surrounding tissues. Within other aspects of the present invention, anti-angiogenic compositions (e.g., in the form of a spray) may be delivered via endoscopic procedures in order to coat tumors, or inhibit angiogenesis in a desired locale. Within yet other aspects of the present invention, surgical meshes which have been coated with anti-angiogenic compositions of the present invention may be utilized in any procedure wherein a surgical mesh might be utilized. For example, within one embodiment of the invention a surgical mesh laden with an anti-angiogenic composition may be utilized during abdominal cancer resection surgery (e.g., subsequent to colon resection) in order to provide support to the structure, and to release an amount of the anti-angiogenic factor.

Within further aspects of the present invention, methods are provided for treating tumor excision sites, comprising administering an anti-angiogenic composition as described above to the resection margins of a tumor subsequent to excision, such that the local recurrence of cancer and the formation of new blood vessels at the site is inhibited. Within one embodiment of the invention, the anti-angiogenic composition(s) (or anti-angiogenic factor(s) alone) are administered directly to the tumor excision site (e.g., applied by swabbing, brushing or otherwise coating the resection margins of the tumor with the anti-angiogenic composition(s) or factor(s)). Alternatively, the anti-angiogenic composition(s) or factor(s) may be incorporated into known surgical pastes prior to administration. Within particularly preferred embodiments of the invention, the anti-angiogenic compositions are applied after hepatic resections for malignancy, and after neurosurgical operations.

Within one aspect of the present invention, anti-angiogenic compositions (as described above) may be administered to the resection margin of a wide variety of tumors, including for example, breast, colon, brain and hepatic tumors. For example, within one embodiment of the invention, anti-angiogenic compositions may be administered to the site of a neurological tumor subsequent to excision, such that the formation of new blood vessels at the site are inhibited. Briefly, the brain is highly functionally localized; i.e., each specific anatomical region is specialized to carry out a specific function. Therefore it is the location of brain pathology that is often more important than the type. A relatively small lesion in a key area can be far more devastating than a much larger lesion in a less important area. Similarly, a lesion on the surface of the brain may be easy to resect surgically, while the same tumor located deep in the brain may not (one would have to cut through too many vital structures to reach it). Also, even benign tumors can be dangerous for several reasons: they may grow in a key area and cause significant damage; even though they

would be cured by surgical resection this may not be possible; and finally, if left unchecked they can cause increased intracranial pressure. The skull is an enclosed space incapable of expansion. Therefore, if something is growing in one location, something else must be being compressed in another location—the result is increased pressure in the skull or increased intracranial pressure. If such a condition is left untreated, vital structures can be compressed, resulting in death. The incidence of CNS (central nervous system) malignancies is 8–16 per 100,000. The prognosis of primary malignancy of the brain is dismal, with a median survival of less than one year, even following surgical resection. These tumors, especially gliomas, are predominantly a local disease which recur within 2 centimeters of the original focus of disease after surgical removal.

Representative examples of brain tumors which may be treated utilizing the compositions and methods described herein include Glial Tumors (such as Anaplastic Astrocytoma, Glioblastoma Multiform, Pilocytic Astrocytoma, Oligodendroglioma, Ependymoma, Myxopapillary Ependymoma, Subependymoma, Choroid Plexus Papilloma); Neuron Tumors (e.g., Neuroblastoma, Ganglioneuroblastoma, Ganglioneuroma, and Medulloblastoma); Pineal Gland Tumors (e.g., Pineoblastoma and Pineocytoma); Meningeal Tumors (e.g., Meningioma, Meningeal Hemangiopericytoma, Meningeal Sarcoma); Tumors of Nerve Sheath Cells (e.g., Schwannoma (Neurolomoma) and Neurofibroma); Lymphomas (e.g., Hodgkin's and Non-Hodgkin's Lymphoma (including numerous subtypes, both primary and secondary); Malignant Tumors (e.g., Craniopharyngioma, Epidermoid Cysts, Dermoid Cysts and Colloid Cysts); and Metastatic Tumors (which can be derived from virtually any tumor, the most common being from lung, breast, melanoma, kidney, and gastrointestinal tract tumors).

INFLAMMATORY ARTHRITIS

Inflammatory arthritis is a serious health problems in developed countries, particularly given the increasing number of aged individuals. For example, one form of inflammatory arthritis, rheumatoid arthritis (RA) is a multisystem chronic, relapsing, inflammatory disease of unknown cause. Although many organs can be affected, RA is basically a severe form of chronic synovitis that sometimes leads to destruction and ankylosis of affected joints (taken from *Robbins Pathological Basis of Disease*, by R. S. Cotran, V. Kumar, and S. L. Robbins, W.B. Saunders Co., 1989). Pathologically the disease is characterized by a marked thickening of the synovial membrane which forms villous projections that extend into the joint space, multilayering of the synovial lining (synovial proliferation), infiltration of the synovial membrane with white blood cells (macrophages, lymphocytes, plasma cells, and lymphoid follicles; called an "inflammatory synovitis"), and deposition of fibrin with cellular necrosis within the synovium. The tissue formed as a result of this process is called pannus and eventually the pannus grows to fill the joint space. The pannus develops an extensive network of new blood vessels through the process of angiogenesis which is essential to the evolution of the synovitis. Release of digestive enzymes [matrix metalloproteinases (e.g., collagenase, stromelysin)] and other mediators of the inflammatory process (e.g., hydrogen peroxide, superoxides, lysosomal enzymes, and products of arachadonic acid metabolism) from the cells of the pannus tissue leads to the progressive destruction of the cartilage tissue. The pannus invades the articular cartilage

leading to erosions and fragmentation of the cartilage tissue. Eventually there is erosion of the subchondral bone with fibrous ankylosis and ultimately bony ankylosis, of the involved joint.

It is generally believed, but not conclusively proven, that RA is an autoimmune disease, and that many different arthrogenic stimuli activate the immune response in the immunogenetically susceptible host. Both exogenous infectious agents (Ebstein-Barr Virus, Rubella virus, Cytomegalovirus, Herpes Virus, Human T-cell Lymphotropic Virus, Mycoplasma, and others) and endogenous proteins (collagen, proteoglycans, altered immunoglobulins) have been implicated as the causative agent which triggers an inappropriate host immune response. Regardless of the inciting agent, autoimmunity plays a role in the progression of the disease. In particular, the relevant antigen is ingested by antigen-presenting cells (macrophages or dendritic cells in the synovial membrane), processed, and presented to T lymphocytes. The T cells initiate a cellular immune response and stimulate the proliferation and differentiation of B lymphocytes into plasma cells. The end result is the production of an excessive inappropriate immune response directed against the host tissues [e.g., antibodies directed against Type II collagen, antibodies directed against the Fc portion of autologous IgG (called "Rheumatoid Factor")]. This further amplifies the immune response and hastens the destruction of the cartilage tissue. Once this cascade is initiated numerous mediators of cartilage destruction are responsible for the progression of rheumatoid arthritis.

Thus, within one aspect of the present invention, methods are provided for treating or preventing inflammatory arthritis (e.g., rheumatoid arthritis) comprising the step of administering to a patient a therapeutically effective amount of an anti-angiogenic factor or anti-angiogenic composition to a joint. Within a preferred embodiment of the invention, anti-angiogenic factors (including anti-angiogenic compositions, as described above) may be administered directly by intra-articular injection, as a surgical paste, or as an oral agent (e.g., containing the anti-angiogenic factor thalidomide). One representative example of such a method is set forth in more detail below in Example 19.

As utilized within the context of the present invention, it should be understood that efficacious administration of the anti-angiogenic factors and compositions described herein may be assessed in several ways, including: (1) by preventing or lessening the pathological and/or clinical symptoms associated with rheumatoid arthritis; (2) by downregulating the white blood cell response which initiates the inflammatory cascade and results in synovitis, swelling, pain, and tissue destruction; (3) by inhibiting the "tumor-like" proliferation of synoviocytes that leads to the development of a locally invasive and destructive pannus tissue; (4) by decreasing the production/activity of matrix metalloproteinases produced by white blood cells, synoviocytes, chondrocytes, and endothelial cells, which degrade the cartilage matrix and result in irreversible destruction of the articular cartilage; and (5) by inhibiting blood vessel formation which provides the framework and nutrients necessary for the growth and development of the pannus tissue. Furthermore, the anti-angiogenic factors or compositions should not be toxic to normal chondrocytes at therapeutic levels. Each of these aspects will be discussed in more detail below.

A. Inflammatory Response

Neutrophils are found in abundance in the synovial fluid, but only in small numbers in the synovial membrane itself.

It is estimated that more than 1 billion neutrophils enter a moderately inflamed rheumatoid knee joint each day (Hollingsworth et al., 1967) and remain there because no pathway exists by which they can leave the joint. These cells release reactive free radicals and lysosomal enzymes which degrade the cartilage tissue. Other PMN products such as prostaglandins and leukotrienes augment the inflammatory response and recruit more inflammatory cells into the joint tissue.

Lymphocytes, particularly T cells, are present in abundance in the diseased synovial tissue. Activated T cells produce a variety of lymphokines and cooperate with B cells to produce autoantibodies. T cells products result in the activation of macrophages, a cell which is thought to have an important role in the pathology of the disease. The macrophages produce a variety of destructive lysosomal enzymes, prostaglandins, and monokines and are also capable of stimulating angiogenesis. One of the more important monokines secreted by macrophages is IL-1. Briefly, IL-1 is known to: stimulate synthesis and release of collagenase by synovial cells and synovial fibroblasts, inhibit proteoglycan synthesis by chondrocytes, activate osteoclasts, induce changes in the endothelium of the synovial vasculature [stimulation of endothelial production of plasminogen activator and colony stimulating factor, expression of leukocyte adhesion molecules, promotion of procoagulant activity (Wider et al., 1991)], and act as a chemoattractant for lymphocytes and neutrophils.

Within one embodiment, downregulation of the white blood cell response, or inhibition of the inflammatory response, may be assessed by determination of the effect of the anti-angiogenic factor or anti-angiogenic composition on the response of neutrophils stimulated with opsonized CPPD crystals or opsonized zirconium. Such methods are illustrated in more detail below in Example 22.

B. Synovial Hyperplasia

During the development of RA, the synovial lining cells become activated by products of inflammation or through phagocytosis of immune complexes. Several subtypes of synovial lining cells have been identified and all of them become intensely activated and undergo excessive hyperplasia and growth when stimulated. As the synovial tissue organizes to form a pannus, the number of synovial cells increases to form a mass 100 times its original size. In many ways, the synovitis in rheumatoid arthritis behaves much like a localized neoplasia (Harris, 1990). In fact, cultured rheumatoid synovial cells develop the phenotypic characteristics of anchorage-independent growth usually associated with neoplastic cells if they given sufficient platelet-derived growth factor (Lafyatis et al., 1989). In addition, the synovial cells also produce large amounts of collagenase, stromelysin, prostaglandins, and Interleukin-1.

The tumor-like proliferation of the cells of the synovial connective tissue stroma (synovial cells, fibroblast-like cells and neovascular tissue) produces a pannus with many features of a localized malignancy. Supporting this tumor analogy are several findings: the pannus expresses high levels of oncoproteins such as c-myc and c-fos, produces metalloproteinases to facilitate surrounding tissue invasion, express cytoskeletal markers characteristic of poorly differentiated mesenchymal tissue (e.g., vimentin); synovial cells in vitro grow rapidly, do not contact inhibit, form foci, and can be grown under anchorage-independent conditions in soft agarose; and pannus tissue is capable of inducing the

growth of a supporting vasculature (i.e. angiogenesis). All these findings are suggestive of a tissue in which normal growth regulation has been lost.

Within one embodiment, inhibition of synovial cell proliferation may be determined by, for example, analysis of ³H-thymidine incorporation into synovial cells, or in vitro synovial cell proliferation. Such methods are illustrated in more detail below in Example 23.

C. Matrix Metalloproteinases (MMP)

Irreparable degradation of the cartilage extracellular matrix is believed to be largely due to the enzymatic action of matrix metalloproteinases on the components of the cartilage matrix. Although numerous other enzymes are likely involved in the development of RA, collagenase (MMP-1) and stromelysin (MMP-3) play an important role (Vincetti et al., 1994) in disease progression. These enzymes are capable of degrading type II collagen and proteoglycans respectively; the 2 major extracellular components of cartilage tissue. Cytokines such as IL-1, epidermal growth factor (EGF), platelet-derived growth factor, and tumor necrosis factor are all potent stimulators of collagenase and stromelysin production. As described above, numerous cell types found in the arthritic joint (white blood cells, synovial cells, endothelial cells, and chondrocytes) are capable of synthesizing and secreting MMPs.

In proliferating rheumatoid synovial tissue, collagenase and stromelysin become the major gene products of the pannus and may comprise as much as 2% of the messenger RNAs produced by the synovial fibroblasts (Brinkerhoff and Auble, 1990). Increased levels of collagenase and stromelysin are present in the cartilage of patients with RA and the level of enzyme activity in the joint correlates well with the severity of the lesion (Martel-Pelletier et al., 1993; Walakovich et al., 1992). Because these enzymes are fundamental to the pathology of RA and result in irreversible cartilage damage, many therapeutic strategies have been devised to inhibit their effects.

Numerous naturally present inhibitors of MMP activity have been identified and named "TIMPs" for Tissue Inhibitors of Metalloproteinases. Many of these protein inhibitors bind with the active MMPs to form 1:1 noncovalent complexes which inactivate the MMP enzymes. The TIMPs are produced locally by chondrocytes and synovial fibroblasts and are likely responsible for the normal regulation of connective tissue degradation. It is thought that much of the damage to the cartilage matrix is due to a local imbalance between MMP and TIMP activity. This is probably due to increased production of metalloproteinases while the production of TIMPs remains at a normal or constant level (Vincetti et al., 1994). To overcome this, therapeutic strategies have been designed to add exogenous TIMPs (e.g., the chemically modified tetracycline molecules, collagen substrate analogues) or to upregulate TIMP production (retinoids, transforming growth factor β , IL-6, IL-11, oncostatin M) in an effort to restore the enzymatic balance. However this approach has yet to translate into significant clinical results.

An alternative approach is to inhibit or downregulate the production of the MMPs to restore a normal balance of activity. Naturally occurring compounds (TNF α , all-trans retinoic acid) and synthetic compounds (retinoids, glucocorticoid hormones) have been demonstrated to inhibit MMP activity by suppressing transcription and synthesis of these proteins. A post-transcriptional method of blocking MMP release could also be expected to result in a decrease in the

amount of MMP produced and an improved balance between MMP and TIMP activity in the joint.

Within one embodiment, a decrease in the production or activity of MMP's may be determined by, for example, analysis of IL-1 induced collagenase expression. One such method is illustrated in more detail below in Example 24.

D. Angiogenesis

The development of an extensive network of new blood vessels is essential to the development of the synovitis present in rheumatoid arthritis (Harris, 1990; Folkman et al., 1989; Sano et al., 1990). Several local mediators such as platelet-derived growth factor (PDGF), TGF- β , and fibroblast growth factor (FGF) are likely responsible for the induction and perpetuation of neovascularization within the synovium. Pannus tissue composed of new capillaries and synovial connective tissue invades and destroys the articular cartilage. The migrating angiogenic vessels themselves produce and secrete increased levels of metalloproteinases such as collagenase and stromelysin capable of degrading the cartilage matrix (Case et al., 1989). The newly formed vessels are also quite "leaky" with gaps present between the microvascular endothelial cells. This facilitates the exudation of plasma proteins into the synovium (which increases swelling), enhances WBCs movement from the circulation into the pannus tissue (which increases inflammation), and leads to the perivascular accumulation of mononuclear inflammatory cells (Wilder et al., 1991).

In summary, the endothelial tissue plays an important role in the development of this disease by expressing the necessary surface receptors to allow inflammatory cells to leave the circulation and enter the developing pannus, secreting proteolytic enzymes capable of degrading the cartilage matrix, and proliferating to form the new vessels (angiogenesis) required for the pannus tissue to increase in size and invade adjacent tissues.

Within one embodiment, inhibition of new blood vessel formation may be readily determined in a variety of assays, including the CAM assay described above and within Example 2.

NEOVASCULAR DISEASES OF THE EYE

As noted above, the present invention also provides methods for treating neovascular diseases of the eye, including for example, corneal neovascularization, neovascular glaucoma, proliferative diabetic retinopathy, retrolental fibroplasia and macular degeneration.

Briefly, corneal neovascularization as a result of injury to the anterior segment is a significant cause of decreased visual acuity and blindness, and a major risk factor for rejection of corneal allografts. As described by Burger et al., *Lab. Invest.* 48:169-180, 1983, corneal angiogenesis involves three phases: a pre-vascular latent period, active neovascularization, and vascular maturation and regression. The identity and mechanism of various angiogenic factors, including elements of the inflammatory response, such as leukocytes, platelets, cytokines, and eicosanoids, or unidentified plasma constituents have yet to be revealed.

Currently no clinically satisfactory therapy exists for inhibition of corneal neovascularization or regression of existing corneal new vessels. Topical corticosteroids appear to have some clinical utility, presumably by limiting stromal inflammation.

Thus, within one aspect of the present invention methods are provided for treating neovascular diseases of the eye

such as corneal neovascularization (including corneal graft neovascularization), comprising the step of administering to a patient a therapeutically effective amount of an anti-angiogenic composition (as described above) to the cornea, such that the formation of blood vessels is inhibited. Briefly, the cornea is a tissue which normally lacks blood vessels. In certain pathological conditions however, capillaries may extend into the cornea from the pericorneal vascular plexus of the limbus. When the cornea becomes vascularized, it also becomes clouded, resulting in a decline in the patient's visual acuity. Visual loss may become complete if the cornea completely opacitates.

Blood vessels can enter the cornea in a variety of patterns and depths, depending upon the process which incites the neovascularization. These patterns have been traditionally defined by ophthalmologists in the following types: pannus trachomatous, pannus leprosus, pannus phlyctenulosus, pannus degenerativus, and glaucomatous pannus. The corneal stroma may also be invaded by branches of the anterior ciliary artery (called interstitial vascularization) which causes several distinct clinical lesions: terminal loops, a "brush-like" pattern, an umbel form, a lattice form, interstitial arcades (from episcleral vessels), and aberrant irregular vessels.

A wide variety of disorders can result in corneal neovascularization, including for example, corneal infections (e.g., trachoma, herpes simplex keratitis, leishmaniasis and onchocerciasis), immunological processes (e.g., graft rejection and Stevens-Johnson's syndrome), alkali burns, trauma, inflammation (of any cause), toxic and nutritional deficiency states, and as a complication of wearing contact lenses.

While the cause of corneal neovascularization may vary, the response of the cornea to the insult and the subsequent vascular ingrowth is similar regardless of the cause. Briefly, the location of the injury appears to be of importance as only those lesions situated within a critical distance of the limbus will incite an angiogenic response. This is likely due to the fact that the angiogenic factors responsible for eliciting the vascular invasion are created at the site of the lesion, and must diffuse to the site of the nearest blood vessels (the limbus) in order to exert their effect. Past a certain distance from the limbus, this would no longer be possible and the limbic endothelium would not be induced to grow into the cornea. Several angiogenic factors are likely involved in this process, many of which are products of the inflammatory response. Indeed, neovascularization of the cornea appears to only occur in association with an inflammatory cell infiltrate, and the degree of angiogenesis is proportional to the extent of the inflammatory reaction. Corneal edema further facilitates blood vessel ingrowth by loosening the corneal stromal framework and providing a pathway of "least resistance" through which the capillaries can grow.

Following the initial inflammatory reaction, capillary growth into the cornea proceeds in the same manner as it occurs in other tissues. The normally quiescent endothelial cells of the limbic capillaries and venules are stimulated to divide and migrate. The endothelial cells project away from their vessels of origin, digest the surrounding basement membrane and the tissue through which they will travel, and migrate towards the source of the angiogenic stimulus. The blind ended sprouts acquire a lumen and then anastomose together to form capillary loops. The end result is the establishment of a vascular plexus within the corneal stroma.

Anti-angiogenic factors and compositions of the present invention are useful by blocking the stimulatory effects of

angiogenesis promoters, reducing endothelial cell division, decreasing endothelial cell migration, and impairing the activity of the proteolytic enzymes secreted by the endothelium.

Within particularly preferred embodiments of the invention, an anti-angiogenic factor may be prepared for topical administration in saline (combined with any of the preservatives and antimicrobial agents commonly used in ocular preparations), and administered in eyedrop form. The anti-angiogenic factor solution or suspension may be prepared in its pure form and administered several times daily. Alternatively, anti-angiogenic compositions, prepared as described above, may also be administered directly to the cornea. Within preferred embodiments, the anti-angiogenic composition is prepared with a muco-adhesive polymer which binds to cornea. Within further embodiments, the anti-angiogenic factors or anti-angiogenic compositions may be utilized as an adjunct to conventional steroid therapy.

Topical therapy may also be useful prophylactically in corneal lesions which are known to have a high probability of inducing an angiogenic response (such as chemical burns). In these instances the treatment, likely in combination with steroids, may be instituted immediately to help prevent subsequent complications.

Within other embodiments, the anti-angiogenic compositions described above may be injected directly into the corneal stroma by an ophthalmologist under microscopic guidance. The preferred site of injection may vary with the morphology of the individual lesion, but the goal of the administration would be to place the composition at the advancing front of the vasculature (i.e., interspersed between the blood vessels and the normal cornea). In most cases this would involve perilimbal corneal injection to "protect" the cornea from the advancing blood vessels. This method may also be utilized shortly after a corneal insult in order to prophylactically prevent corneal neovascularization. In this situation the material could be injected in the perilimbal cornea interspersed between the corneal lesion and its undesired potential limbal blood supply. Such methods may also be utilized in a similar fashion to prevent capillary invasion of transplanted corneas. In a sustained-release form injections might only be required 2-3 times per year. Asteroid could also be added to the injection solution to reduce inflammation resulting from the injection itself.

Within another aspect of the present invention, methods are provided for treating neovascular glaucoma, comprising the step of administering to a patient a therapeutically effective amount of an anti-angiogenic composition to the eye, such that the formation of blood vessels is inhibited.

Briefly, neovascular glaucoma is a pathological condition wherein new capillaries develop in the iris of the eye. The angiogenesis usually originates from vessels located at the pupillary margin, and progresses across the root of the iris and into the trabecular meshwork. Fibroblasts and other connective tissue elements are associated with the capillary growth and a fibrovascular membrane develops which spreads across the anterior surface of the iris. Eventually this tissue reaches the anterior chamber angle where it forms synechiae. These synechiae in turn coalesce, scar, and contract to ultimately close off the anterior chamber angle. The scar formation prevents adequate drainage of aqueous humor through the angle and into the trabecular meshwork, resulting in an increase in intraocular pressure that may result in blindness.

Neovascular glaucoma generally occurs as a complication of diseases in which retinal ischemia is predominant. In

particular, about one third of the patients with this disorder have diabetic retinopathy and 28% have central retinal vein occlusion. Other causes include chronic retinal detachment, end-stage glaucoma, carotid artery obstructive disease, retrolental fibroplasia, sickle-cell anemia, intraocular tumors, and carotid cavernous fistulas. In its early stages, neovascular glaucoma may be diagnosed by high magnification slitlamp biomicroscopy, where it reveals small, dilated, disorganized capillaries (which leak fluorescein) on the surface of the iris. Later gonioscopy demonstrates progressive obliteration of the anterior chamber angle by fibrovascular bands. While the anterior chamber angle is still open, conservative therapies may be of assistance. However, once the angle closes surgical intervention is required in order to alleviate the pressure.

Therefore, within one embodiment of the invention anti-angiogenic factors (either alone or in an anti-angiogenic composition, as described above) may be administered topically to the eye in order to treat early forms of neovascular glaucoma.

Within other embodiments of the invention, anti-angiogenic compositions may be implanted by injection of the composition into the region of the anterior chamber angle. This provides a sustained localized increase of anti-angiogenic factor, and prevents blood vessel growth into the area. Implanted or injected anti-angiogenic compositions which are placed between the advancing capillaries of the iris and the anterior chamber angle can "defend" the open angle from neovascularization. As capillaries will not grow within a significant radius of the anti-angiogenic composition, patency of the angle could be maintained. Within other embodiments, the anti-angiogenic composition may also be placed in any location such that the anti-angiogenic factor is continuously released into the aqueous humor. This would increase the anti-angiogenic factor concentration within the humor, which in turn bathes the surface of the iris and its abnormal capillaries, thereby providing another mechanism by which to deliver the medication. These therapeutic modalities may also be useful prophylactically and in combination with existing treatments.

Within another aspect of the present invention, methods are provided for treating proliferative diabetic retinopathy, comprising the step of administering to a patient a therapeutically effective amount of an anti-angiogenic composition to the eyes, such that the formation of blood vessels is inhibited.

Briefly, the pathology of diabetic retinopathy is thought to be similar to that described above for neovascular glaucoma. In particular, background diabetic retinopathy is believed to convert to proliferative diabetic retinopathy under the influence of retinal hypoxia. Generally, neovascular tissue sprouts from the optic nerve (usually within 10 mm of the edge), and from the surface of the retina in regions where tissue perfusion is poor. Initially the capillaries grow between the inner limiting membrane of the retina and the posterior surface of the vitreous. Eventually, the vessels grow into the vitreous and through the inner limiting membrane. As the vitreous contracts, traction is applied to the vessels, often resulting in shearing of the vessels and blinding of the vitreous due to hemorrhage. Fibrous traction from scarring in the retina may also produce retinal detachment.

The conventional therapy of choice is panretinal photocoagulation to decrease retinal tissue, and thereby decrease retinal oxygen demands. Although initially effective, there is a high relapse rate with new lesions forming in other parts of the retina. Complications of this therapy include a

decrease in peripheral vision of up to 50% of patients, mechanical abrasions of the cornea, laser-induced cataract formation, acute glaucoma, and stimulation of subretinal neovascular growth (which can result in loss of vision). As a result, this procedure is performed only when several risk factors are present, and the risk-benefit ratio is clearly in favor of intervention.

Therefore, within particularly preferred embodiments of the invention, proliferative diabetic retinopathy may be treated by injection of an anti-angiogenic factor(s) (or anti-angiogenic composition) into the aqueous humor or the vitreous, in order to increase the local concentration of anti-angiogenic factor in the retina. Preferably, this treatment should be initiated prior to the acquisition of severe disease requiring photocoagulation. Within other embodiments of the invention, arteries which feed the neovascular lesions may be embolized (utilizing anti-angiogenic compositions, as described above).

Within another aspect of the present invention, methods are provided for treating retrolental fibroplasia, comprising the step of administering to a patient a therapeutically effective amount of an anti-angiogenic factor (or anti-angiogenic composition) to the eye, such that the formation of blood vessels is inhibited.

Briefly, retrolental fibroplasia is a condition occurring in premature infants who receive oxygen therapy. The peripheral retinal vasculature, particularly on the temporal side, does not become fully formed until the end of fetal life. Excessive oxygen (even levels which would be physiologic at term) and the formation of oxygen free radicals are thought to be important by causing damage to the blood vessels of the immature retina. These vessels constrict, and then become structurally obliterated on exposure to oxygen. As a result, the peripheral retina fails to vascularize and retinal ischemia ensues. In response to the ischemia, neovascularization is induced at the junction of the normal and the ischemic retina.

In 75% of the cases these vessels regress spontaneously. However, in the remaining 25% there is continued capillary growth, contraction of the fibrovascular component, and traction on both the vessels and the retina. This results in vitreous hemorrhage and/or retinal detachment which can lead to blindness. Neovascular angle-closure glaucoma is also a complication of this condition.

As it is often impossible to determine which cases will spontaneously resolve and which will progress in severity, conventional treatment (i.e., surgery) is generally initiated only in patients with established disease and a well developed pathology. This "wait and see" approach precludes early intervention, and allows the progression of disease in the 25% who follow a complicated course. Therefore, within one embodiment of the invention, topical administration of anti-angiogenic factors (or anti-angiogenic compositions, as described above) may be accomplished in infants which are at high risk for developing this condition in an attempt to cut down on the incidence of progression of retrolental fibroplasia. Within other embodiments, intravitreal injections and/or intraocular implants of an anti-angiogenic composition may be utilized. Such methods are particularly preferred in cases of established disease, in order to reduce the need for surgery.

OTHER THERAPEUTIC USES OF ANTI-ANGIOGENIC COMPOSITIONS

Anti-angiogenic factors and compositions of the present invention may be utilized in a variety of additional methods

in order to therapeutically treat a cancer or tumor. For example, anti-angiogenic factors or compositions described herein may be formulated for topical delivery, in order to treat cancers such as skin cancer, head and neck tumors, breast tumors, and Kaposi's sarcoma. Within yet other aspects, the anti-angiogenic factors or compositions provided herein may be utilized to treat superficial forms of bladder cancer by, for example, intravesical administration.

In addition to cancer, however, numerous other non-tumorigenic angiogenesis-dependent diseases which are characterized by the abnormal growth of blood vessels may also be treated with the anti-angiogenic factors or compositions of the present invention. Representative examples of such non-tumorigenic angiogenesis-dependent diseases include hypertrophic scars and keloids, proliferative diabetic retinopathy (discussed above), rheumatoid arthritis (discussed above), arteriovenous malformations (discussed above), atherosclerotic plaques, delayed wound healing, hemophilic joints, nonunion fractures, Osier-Weber syndrome, psoriasis, pyogenic granuloma, scleroderma, trachoma, menorrhagia (discussed above) and vascular adhesions.

For example, within one aspect of the present invention methods are provided for treating hypertrophic scars and keloids, comprising the step of administering one of the above-described anti-angiogenic compositions to a hypertrophic scar or keloid.

Briefly, healing of wounds and scar formation occurs in three phases: inflammation, proliferation, and maturation. The first phase, inflammation, occurs in response to an injury which is severe enough to break the skin. During this phase, which lasts 3 to 4 days, blood and tissue fluid form an adhesive coagulum and fibrinous network which serves to bind the wound surfaces together. This is then followed by a proliferative phase in which there is ingrowth of capillaries and connective tissue from the wound edges, and closure of the skin defect. Finally, once capillary and fibroblastic proliferation has ceased, the maturation process begins wherein the scar contracts and becomes less cellular, less vascular, and appears flat and white. This final phase may take between 6 and 12 months.

If too much connective tissue is produced and the wound remains persistently cellular, the scar may become red and raised. If the scar remains within the boundaries of the original wound it is referred to as a hypertrophic scar, but if it extends beyond the original scar and into the surrounding tissue, the lesion is referred to as a keloid. Hypertrophic scars and keloids are produced during the second and third phases of scar formation. Several wounds are particularly prone to excessive endothelial and fibroblastic proliferation, including burns, open wounds, and infected wounds. With hypertrophic scars, some degree of maturation occurs and gradual improvement occurs. In the case of keloids however, an actual tumor is produced which can become quite large. Spontaneous improvement in such cases rarely occurs.

Therefore, within one embodiment of the present invention either anti-angiogenic factors alone, or anti-angiogenic compositions as described above, are directly injected into a hypertrophic scar or keloid, in order to prevent the progression of these lesions. The frequency of injections will depend upon the release kinetics of the polymer used (if present), and the clinical response. This therapy is of particular value in the prophylactic treatment of conditions which are known to result in the development of hypertrophic scars and keloids (e.g., burns), and is preferably initiated after the proliferative phase has had time to

progress (approximately 14 days after the initial injury), but before hypertrophic scar or keloid development.

As noted above, within yet another aspect of the present invention, vascular grafts are provided comprising a synthetic tube, the surface of which is coated with an anti-angiogenic composition as described above. Briefly, vascular grafts are synthetic tubes, usually made of Dacron or Gortex, inserted surgically to bypass arterial blockages, most frequently from the aorta to the femoral, or the femoral to the popliteal artery. A major problem which particularly complicates femoral-popliteal bypass grafts is the formation of a subendothelial scar-like reaction in the blood vessel wall called neointimal hyperplasia, which narrows the lumen within and adjacent to either end of the graft, and which can be progressive. A graft coated with or containing anti-angiogenic factors (or anti-angiogenic compositions, as described above) may be utilized to limit the formation of neointimal hyperplasia at either end of the graft. The graft may then be surgically placed by conventional bypass techniques.

Anti-angiogenic compositions of the present invention may also be utilized in a variety of other manners. For example, they may be incorporated into surgical sutures in order to prevent stitch granulomas, implanted in the uterus (in the same manner as an IUD) for the treatment of menorrhagia or as a form of female birth control, administered as either a peritoneal lavage fluid or for peritoneal implantation in the treatment of endometriosis, attached to a monoclonal antibody directed against activated endothelial cells as a form of systemic chemotherapy, or utilized in diagnostic imaging when attached to a radioactively labeled monoclonal antibody which recognizes activated endothelial cells.

FORMULATION AND ADMINISTRATION

As noted above, anti-angiogenic compositions of the present invention may be formulated in a variety of forms (e.g., microspheres, pastes, films or sprays). Further, the compositions of the present invention may be formulated to contain more than one anti-angiogenic factor, to contain a variety of additional compounds, to have certain physical properties (e.g., elasticity, a particular melting point, or a specified release rate). Within certain embodiments of the invention, compositions may be combined in order to achieve a desired effect (e.g., several preparations of microspheres may be combined in order to achieve both a quick and a slow or prolonged release of one or more anti-angiogenic factor).

Anti-angiogenic factors and compositions of the present invention may be administered either alone, or in combination with pharmaceutically or physiologically acceptable carrier, excipients or diluents. Generally, such carriers should be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the therapeutic agent with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrans, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with nonspecific serum albumin are exemplary appropriate diluents.

As noted above, anti-angiogenic factors, anti-angiogenic compositions, or pharmaceutical compositions provided herein may be prepared for administration by a variety of different routes, including for example intrarticularly,

intraocularly, intranasally, intradermally, sublingually, orally, topically, intravesically, intrathecally, topically, intravenously, intraperitoneally, intracranially, intramuscularly, subcutaneously, or even directly into a tumor or disease site. Other representative routes of administration include gastroscopy, ECRP and colonoscopy, which do not require full operating procedures and hospitalization, but may require the presence of medical personnel.

The anti-angiogenic factors, anti-angiogenic compositions and pharmaceutical compositions provided herein may be placed within containers, along with packaging material which provides instructions regarding the use of such materials. Generally, such instructions will include a tangible expression describing the reagent concentration, as well as within certain embodiments, relative amounts of excipient ingredients or diluents (e.g., water, saline or PBS) which may be necessary to reconstitute the anti-angiogenic factor, anti-angiogenic composition, or pharmaceutical composition.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

Example 1

Preparation of Anti-Invasive Factor

The shoulder girdle and skull from a dogfish is excised, then scraped with a scalpel in order to remove all muscle and associated connective tissue from the cartilage. The cartilage is then homogenized with a tissue grinder, and extracted by continuous stirring at room temperature for 2 to 5 days in a solution containing 2.0M guanidium hydrochloride and 0.02M MES at pH 6.0.

After 2 to 5 days, the cartilage extract is passed through gauze netting in order to remove the larger constituents. The filtrate is then passed through an Amicon ultrafiltration unit which utilizes spiral-wound cartridges, with a molecular weight cutoff of 100,000. The filtrate (containing proteins with a molecular weight of less than 100,000 daltons) is then dialyzed against 0.02M MES buffer (pH 6) with an Amicon ultrafiltration unit which retains proteins with a molecular weight of greater than 3,000 daltons. Utilizing this method, low molecular weight proteins and constituents are removed, as well as excessive amounts of guanidium HCl. The dialysate is concentrated to a final concentration 9 mg/ml.

Example 2

Analysis of Various Agents for Anti-Angiogenic Activity

A. Chick Chorioallantoic Membrane ("Cam") Assays

Fertilized, domestic chick embryos were incubated for 3 days prior to shell-less culturing. In this procedure, the egg contents were emptied by removing the shell located around the air space. The interior shell membrane was then severed and the opposite end of the shell was perforated to allow the contents of the egg to gently slide out from the blunted end. The egg contents were emptied into round-bottom sterilized glass bowls and covered with petri dish covers. These were then placed into an incubator at 90% relative humidity and 3% CO₂ and incubated for 3 days.

Paclitaxel (Sigma, St. Louis, Mich.) was mixed at concentrations of 1, 5, 10, 30 µg per 10 ml aliquot of 0.5%

aqueous methylcellulose. Since paclitaxel is insoluble in water, glass beads were used to produce fine particles. Ten microliter aliquots of this solution were dried on parafilm for 1 hour forming disks 2 mm in diameter. The dried disks containing paclitaxel were then carefully placed at the growing edge of each CAM at day 6 of incubation. Controls were obtained by placing paclitaxel-free methylcellulose disks on the CAMs over the same time course. After a 2 day exposure (day 8 of incubation) the vasculature was examined with the aid of a stereomicroscope. Liposyn II, a white opaque solution, was injected into the CAM to increase the visibility of the vascular details. The vasculature of unstained, living embryos were imaged using a Zeiss stereomicroscope which was interfaced with a video camera (Dage-MTI Inc., Michigan City, Ind.). These video signals were then displayed at 160 times magnification and captured using an image analysis system (Vidas, Kontron; Etching, Germany). Image negatives were then made on a graphics recorder (Model 3000; Matrix Instruments, Orangeburg, N.Y.).

The membranes of the 8 day-old shell-less embryo were flooded with 2% glutaraldehyde in 0.1M Na cacodylate buffer; additional fixative was injected under the CAM. After 10 minutes in situ, the CAM was removed and placed into fresh fixative for 2 hours at room temperature. The tissue was then washed overnight in cacodylate buffer containing 6% sucrose. The areas of interest were postfixed in 1% osmium tetroxide for 1.5 hours at 4° C. The tissues were then dehydrated in a graded series of ethanol, solvent exchanged with propylene oxide, and embedded in Spurr resin. Thin sections were cut with a diamond knife, placed on copper grids, stained, and examined in a Joel 1200EX electron microscope. Similarly, 0.5 nm sections were cut and stained with toluidine blue for light microscopy.

At day 11 of development, chick embryos were used for the corrosion casting technique. Mercor resin (Ted Pella, Inc., Redding, Calif.) was injected into the CAM vasculature using a 30-gauge hypodermic needle. The casting material consisted of 2.5 grams of Mercor CL-2B polymer and 0.05 grams of catalyst (55% benzoyl peroxide) having a 5 minute polymerization time. After injection, the plastic was allowed to sit in situ for an hour at room temperature and then overnight in an oven at 65° C. The CAM was then placed in 50% aqueous solution of sodium hydroxide to digest all organic components. The plastic casts were washed extensively in distilled water, air-dried, coated with gold/palladium, and viewed with the Philips 501B scanning electron microscope.

Results of the above experiments are shown in FIGS. 1-4. Briefly, the general features of the normal chick shell-less egg culture are shown in FIG. 1A. At day 6 of incubation, the embryo is centrally positioned to a radially expanding network of blood vessels; the CAM develops adjacent to the embryo. These growing vessels lie close to the surface and are readily visible making this system an idealized model for the study of angiogenesis. Living, unstained capillary networks of the CAM can be imaged noninvasively with a stereomicroscope. FIG. 1B illustrates such a vascular area in which the cellular blood elements within capillaries were recorded with the use of a video/computer interface. The 3-dimensional architecture of such CAM capillary networks is shown by the corrosion casting method and viewed in the scanning electron microscope (FIG. 1C). These castings revealed underlying vessels which project toward the CAM surface where they form a single layer of anastomotic capillaries.

Transverse sections through the CAM show an outer ectoderm consisting of a double cell layer, a broader meso-

dermal layer containing capillaries which lie subjacent to the ectoderm, adventitial cells, and an inner, single endodermal cell layer (FIG. 1D). At the electron microscopic level, the typical structural details of the CAM capillaries are demonstrated. Typically, these vessels lie in close association with the inner cell layer of ectoderm (FIG. 1E).

After 48 hours exposure to paclitaxel at concentrations of 0.25, 0.5, 1, 5, 10, or 30 ug, each CAM was examined under living conditions with a stereomicroscope equipped with a video/computer interface in order to evaluate the effects on angiogenesis. This imaging setup was used at a magnification of 160 times which permitted the direct visualization of blood cells within the capillaries; thereby blood flow in areas of interest could be easily assessed and recorded. For this study, the inhibition of angiogenesis was defined as an area of the CAM lacking a capillary network and vascular blood flow. Throughout the experiments, avascular zones were assessed on a 4 point avascular gradient (Table I). This scale represents the degree of overall inhibition with maximal inhibition represented as a 3 on the avascular gradient scale. Paclitaxel was very consistent and induced a maximal avascular zone (6 mm in diameter or a 3 on the avascular gradient scale) within 48 hours depending on its concentration.

TABLE I

AVASCULAR GRADIENT

0 - normal vascularity
1 - lacking some microvascular movement
2* - small avascular zone approximately 2 mm in diameter
3* - avascularity extending beyond the disk (6 mm in diameter)

The dose-dependent, experimental data of the effects of paclitaxel at different concentrations are shown in Table II.

TABLE II

Angiogenic Inhibition by Paclitaxel

Paclitaxel in Methylcellulose Disks (ug)	Embryos Evaluated (positive/total)	% Inhibition
0.25	2/11	18
0.5	6/11	54
1	6/15	40
5	20/27	76
10	16/21	76
30	31/31	100
0 (control)	0/40	0

TABLE III

Angiogenic Inhibition of Paclitaxel-Loaded Thermopaste

Paclitaxel-loaded Thermopaste (%)	Embryos Evaluated (positive/n)
0.25	4/4
0.5	4/4
1	8/8
5	4/4
10	5/5
20	6/6
0 (control)	0/30

Typical paclitaxel-treated CAMs are also shown with the transparent methylcellulose disk centrally positioned over the avascular zone measuring 6 mm in diameter. At a slightly higher magnification, the periphery of such avascular zones is clearly evident (FIG. 2C); the surrounding functional

vessels were often redirected away from the source of paclitaxel (FIGS. 2C and 2D). Such angular redirecting of blood flow was never observed under normal conditions. Another feature of the effects of paclitaxel was the formation of blood islands within the avascular zone representing the aggregation of blood cells.

The associated morphological alterations of the paclitaxel-treated CAM are readily apparent at both the light and electron microscopic levels. For the convenience of presentation, three distinct phases of general transition from the normal to the avascular state are shown. Near the periphery of the avascular zone the CAM is hallmarked by an abundance of mitotic cells within all three germ layers (FIGS. 3A and 4A). This enhanced mitotic division was also a consistent observation for capillary endothelial cells. However, the endothelial cells remained junctionally intact with no extravasation of blood cells. With further degradation, the CAM is characterized by the breakdown and dissolution of capillaries (FIGS. 3B and 4B). The presumptive endothelial cells, typically arrested in mitosis, still maintain a close spatial relationship with blood cells and lie subjacent to the ectoderm; however, these cells are not junctionally linked. The most central portion of the avascular zone was characterized by a thickened ectodermal and endodermal layer (FIGS. 3C and 4C). Although these layers were thickened, the cellular junctions remained intact and the layers maintained their structural characteristics. Within the mesoderm, scattered mitotically arrested cells were abundant; these cells did not exhibit the endothelial cell polarization observed in the former phase. Also, throughout this avascular region, degenerating cells were common as noted by the electron dense vacuoles and cellular debris (FIG. 4C).

In summary, this study demonstrated that 48 hours after paclitaxel application to the CAM, angiogenesis was inhibited. The blood vessel inhibition formed an avascular zone which was represented by three transitional phases of paclitaxel's effect. The central, most affected area of the avascular zone contained disrupted capillaries with extravasated red blood cells; this indicated that intercellular junctions between endothelial cells were absent. The cells of the endoderm and ectoderm maintained their intercellular junctions and therefore these germ layers remained intact; however, they were slightly thickened. As the normal vascular area was approached, the blood vessels retained their junctional complexes and therefore also remained intact. At the periphery of the paclitaxel-treated zone, further blood vessel growth was inhibited which was evident by the typical redirecting or "elbowing" effect of the blood vessels (FIG. 2D).

Paclitaxel-treated avascular zones also revealed an abundance of cells arrested in mitosis in all three germ layers of the CAM; this was unique to paclitaxel since no previous study has illustrated such an event. By being arrested in mitosis, endothelial cells could not undergo their normal metabolic functions involved in angiogenesis. In comparison, the avascular zone formed by suramin and cortisone acetate do not produce mitotically arrested cells in the CAM; they only prevented further blood vessel growth into the treated area. Therefore, even though these agents are anti-angiogenic, there are many points in which the angiogenesis process may be targeted.

The effects of paclitaxel over the 48 hour duration were also observed. During this period of observation it was noticed that inhibition of angiogenesis occurs as early as 9 hours after application. Histological sections revealed a similar morphology as seen in the first transition phase of the

avascular zone at 48 hours illustrated in FIGS. 3A and 4A. Also, we observed in the revascularization process into the avascular zone previously observed. It has been found that the avascular zone formed by heparin and angiostatic steroids became revascularized 60 hours after application. In one study, paclitaxel-treated avascular zones did not revascularize for at least 7 days after application implying a more potent long-term effect.

Example 3

Encapsulation of Suramin

One milliliter of 5% ELVAX (poly(ethylene-vinyl acetate) cross-linked with 5% vinyl acetate) in dichloromethane ("DCM") is mixed with a fixed weight of sub-micron ground sodium suramin. This mixture is injected into 5 ml of 5% Polyvinyl Alcohol ("PVA") in water in a 30 ml flat bottomed test tube. Tubes containing different weights of the drug are then suspended in a multi-sample water bath at 40° for 90 minutes with automated stirring. The mixtures are removed, and microsphere samples taken for size analysis. Tubes are centrifuged at 1000 g for 5 min. The PVA supernatant is removed and saved for analysis (nonencapsulated drug). The microspheres are then washed (vortexed) in 5 ml of water and recentrifuged. The 5 ml wash is saved for analysis (surface bound drug). Microspheres are then wetted in 50 ul of methanol, and vortexed in 1 ml of DCM to dissolve the ELVAX. The microspheres are then warmed to 40° C., and 5 ml of 50° C. water is slowly added with stirring. This procedure results in the immediate evaporation of DCM, thereby causing the release of sodium suramin into the 5 ml of water.

All samples were assayed for drug content by quantification of fluorescence. Briefly, sodium suramin absorbs uv/vis with a lambda max of 312 nm. This absorption is linear in the 0 to 100 ug/ml range in both water and 5% PVA. Sodium suramin also fluoresces strongly with an excitation maximum at 312 nm, and emission maximum at 400 nm. This fluorescence is quantifiable in the 0 to 25 ug/ml range.

The results of these experiments is shown in FIGS. 5-11. Results are shown in FIGS. 5-10. Briefly, the size distribution of microspheres by number (FIG. 5) or by weight (FIG. 6) appears to be unaffected by inclusion of the drug in the DCM. Good yields of microspheres in the 20 to 60 µm range may be obtained.

The encapsulation of suramin is very low (<1%) (see FIG. 8). However as the weight of drug is increased in the DCM the total amount of drug encapsulated increased although the % encapsulation decreased. As is shown in FIG. 7, 50 ug of drug may be encapsulated in 50 mg of ELVAX. Encapsulation of sodium suramin in 2.5% PVA containing 10% NaCl is shown in FIG. 9 (size distribution by weight). Encapsulation of sodium suramin in 5% PVA containing 10% NaCl is shown in FIGS. 10 and 11 (size distribution by weight, and number, respectively).

To assess suramin and cortisone acetate as potential anti-angiogenic agents, each agent was mixed with 0.5% methylcellulose and applied the dried disks containing the agent onto the developing blood vessels of the 6-day old CAM. A combination treatment of suramin (70 µg) with cortisone acetate (20 µg) was successful in inhibiting angiogenesis when tested on the CAM for 48 hours. The resulting avascular region measured 6 mm in diameter and revealed an absence of blood flow and the appearance of sparse blood islands (FIGS. 28A and 28B).

Example 4

Encapsulation of Paclitaxel

Five hundred micrograms of either paclitaxel or baccatin (a paclitaxel analog, available from Inflazyme Pharmaceu-

ticals Inc., Vancouver, British Columbia, Canada) are dissolved in 1 ml of a 50:50 ELVAX:poly-L-lactic acid mixture in dcm. Microspheres are then prepared in a dissolution machine (Six-spindle dissolution tester, VanderKamp, Van Kell Industries Inc., U.S.A.) in triplicate at 200 rpm, 42° C., for 3 hours. Microspheres so prepared are washed twice in water and sized on the microscope.

Determination of paclitaxel encapsulation is undertaken in a uv/vis assay (uv/vis lambda max. at 237 nm, fluorescence assay at excitation 237, emission at 325 nm; Fluorescence results are presented in square brackets []). Utilizing the procedures described above, 58 µg (+/-12 µg) [75 µg (+/-25 µg)] of paclitaxel may be encapsulated from a total 500 µg of starting material. This represents 12% (+/-2.4%) [15% (+/-5%)] of the original weight, or 1.2% (+/-0.25%) [1.5% (+/-0.5%)] by weight of the polymer. After 18 hours of tumbling in an oven at 37° C., 10.3% (+/-10%) [6% (+/-5.6%)] of the total paclitaxel had been released from the microspheres.

For baccatin, 100 +/- 15 µg [83 +/- 231 g] of baccatin can be encapsulated from a total of 500 µg starting material. This represents a 20% (+/-3%) [17% (+/-5%)] of the original weight of baccatin, and 2% (+/-0.3%) [1.7% (+/-0.5%)] by weight of the polymer. After 18 hours of tumbling in an oven at 37° C., 55% (+/-13%) [60% (+/-23%)] of the baccatin is released from the microspheres.

Example 5

Analysis of Surgical Paste Containing Anti-Angiogenic Compositions

Fisher rats weighing approximately 300 grams are anesthetized, and a 1 cm transverse upper abdominal incision is made. Two-tenths of a milliliter of saline containing 1x10⁶ live 9 L gliosarcoma cells (eluted immediately prior to use from tissue culture) are injected into 2 of the 5 hepatic lobes by piercing a 27 gauge needle 1 cm through the liver capsule. The abdominal wound is closed with 6.0 resorbable suture and skin clips and the GA terminated.

After 2 weeks, the tumor deposits will measure approximately 1 cm. At this time, both hepatic tumors are resected and the bare margin of the liver is packed with a hemostatic agent. The rats are divided into two groups: half is administered polymeric carrier alone, and the other half receives an anti-angiogenic composition.

Rats are sacrificed 2, 7, 14, 21 and 84 days post hepatic resection. In particular, the rats are euthanized by injecting Euthanyl into the dorsal vein of the tail. The liver, spleen, and both lungs are removed, and histologic analysis is performed in order to study the tumors for evidence of anti-angiogenic activity.

Example 6

Embolization of Rat Arteries

Fisher rats weighing approximately 300 grams are anesthetized. Utilizing aseptic procedures, a 1 cm transverse upper abdominal incision is made, and the liver identified. Two-tenths of a milliliter of saline containing 1 million live 9 L gliosarcoma cells (eluted immediately prior from tissue culture) is injected into each of the 5 hepatic lobes by piercing a 27 gauge needle 1 cm through the liver capsule. One-tenth of a milliliter of normal saline is injected into the needle as it is withdrawn to ensure that there is no spillage of cells into the peritoneal cavity. A pledger of gelfoam is placed on each of the puncture sites to ensure hemostasis.

The abdominal wound is closed with 6.0 resorbable suture with skin clips, and the anesthetic terminated. The rat is returned to the animal care facility to have a standard diet for 14 days, at which time each tumor deposit will measure 1 cm in diameter. The same procedure is repeated using Westar rats and a Colon Cancer cell line (Radiologic Oncology Lab, M. D. Anderson, Houston, Tex.). In this instance, 3 weeks are required post-injection for the tumor deposits to measure 1 cm in diameter each.

After 2 or 3 weeks, depending on the rat species, the same general anesthetic procedure is followed and a midline abdominal incision is performed. The duodenum is flipped and the gastroduodenal artery is identified and mobilized. Ties are placed above and below a cutdown site on the midportion of the gastroduodenal artery (GDA), and 0.038 inch polyethylene tubing is introduced in a retrograde fashion into the artery using an operating microscope. The tie below the insertion point will ligate the artery, while the one above will fix the catheter in place. Angiography is performed by injecting 0.5 ml of 60% radiopaque contrast material through the catheter as an x-ray is taken. The hepatic artery is then embolized by refluxing particles measuring 15-200 µm through the gastroduodenal artery catheter until flow, observed via the operating microscope, is seen to cease for at least 30 seconds. Occlusion of the hepatic artery is confirmed by repeating an angiogram through the GDA catheter. Utilizing this procedure, one-half of the rats receive 15-200 µm particles of polymer alone, and the other half receive 15-200 µm particles of the polymer-anti-angiogenic factor composition. The upper GDA ligature is tightened to occlude the GDA as the catheter is withdrawn to ensure hemostasis, and the hepatic artery (although embolized) is left intact. The abdomen is closed with 6.0 absorbable suture and surgical clips.

The rats are subsequently sacrificed at 2, 7, 14, 21 and 84 days post-embolization in order to determine efficacy of the anti-angiogenic factor. Briefly, general anesthetic is given, and utilizing aseptic precautions, a midline incision is performed. The GDA is mobilized again, and after placing a ligature near the junction of the GDA and the hepatic artery (i.e., well above the site of the previous cutdown), a 0.038-inch polyethylene tubing is inserted via cutdown of the vessel and angiography is performed. The rat is then euthanized by injecting Euthanyl into the dorsal vein of the tail. Once euthanasia is confirmed, the liver is removed en bloc along with the stomach, spleen and both lungs.

Histologic analysis is performed on a prepared slide stained with hematoxylin and eosin ("H and E") stain. Briefly, the lungs are sectioned at 1 cm intervals to assess passage of embolic material through the hepatic veins and into the right side of circulation. The stomach and spleen are also sectioned in order to assess inadvertent immobilization from reflux of particles into the celiac access of the collateral circulation.

Example 7

Transplantation of Biliary Stents in Rats

General anesthetic is administered to 300 gram Fisher rats. A 1 cm transverse incision is then made in the upper abdomen, and the liver identified. In the most superficial lobe, 0.2 ml of saline containing 1 million cells of 9 L gliosarcoma cells (eluted from tissue culture immediately prior to use) is injected via a 27 gauge needle to a depth of 1 cm into the liver capsule. Hemostasis is achieved after removal of the needle by placing a pledger of gelfoam at the